Review of Literature
2.1 Pain
Any stimulus causing or having potential to cause injury leads to pain. It is defined as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” by International Association for the Study of Pain (IASP). Pain is evoked as a defense mechanism against noxious stimuli which warns and instructs the individual to withdraw from damaging situations, to protect a damaged body part while it heals, and to avoid similar experiences in the future. However, unpleasant feeling of pain is a terrible fear for mankind. Therefore relief from pain is a primary duty of a physician.
Chronic painful situation is typical for a large number of diseases, and pain intensity is often used by the patients and health professionals to evaluate the progression of the disease or success of the therapy. Tremendous growth in pain therapy has improved the quality of life; however selection of perfect pain relievers is still a challenge due to their severe side effects. Challenge of pain treatment mostly lies on complexity of pain physiology. Therefore in-depth analysis of the mechanism of pain perception and identification of potential therapeutical targets is needed for successful treatment of pain. Further, search of pain relievers with minimal or no side effects is demand of the day.

2.2 Nociceptors
Painful stimuli are received by specialized afferent neurons, known as nociceptors. A nociceptor is a peripheral sensory nerve fiber that responds to damaging or potentially damaging stimuli by sending signals to the spinal cord and brain (Basbaum and Jessell, 2000). Nociception is the process by which intense thermal, mechanical, or chemical stimuli are detected by nociceptors.

2.2.1 Anatomy of nociceptors
Primary afferent nociceptive fibers have a unique, pseudo-unipolar morphology. Free terminals of nociceptors are distributed throughout the body, which are activated by noxious stimuli and action potential travels up to trigeminal ganglion for the face or to dorsal root ganglia (DRG) for rest of the body. The peripheral and central axonal branches from cell body innervate their target organ and the spinal cord, respectively. The majority of proteins synthesized by the DRG or trigeminal ganglia cells are distributed to both central and peripheral terminals. Therefore, central and peripheral
terminals of these neurons are biochemically equivalent which enables them to receive and send messages from either end. This antidromic stimulation is unique property of nociceptors in contrast to normal orthodromic stimulation of neurons.

There are two major classes of nociceptors (Meyer et al., 2008). One class includes nociceptors having thinly myelinated, medium-diameter ‘Aδ fibers’ with 5-36 m/s conduction velocity that mediate acute and well-localized first pain. Nociceptors of the other class have small-diameter, unmyelinated ‘C fibers’ with 0.5-2.0 m/s conduction velocity that convey poorly localized, second or slow pain. However, there are different ‘Aβ fibers’ which respond to innocuous mechanical stimulation (i.e., light touch). C fibers may further be categorized into two groups. Peptidergic C fibers contain pro-inflammatory peptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) and are regulated by nerve growth factor. The other nonpeptidergic C fibers contain a distinctive phosphatase called thiamine monophosphatase or fluoride-resistant acid phosphatase (FRAP+) and are sensitive to GDNF, produced by the Schwann cells via a common specific receptor called tyrosine-kinase (Coutaux et al., 2005). Nonpeptidergic C fibers may be identified by isolectin B4 (IB4) immunoreactivity (Snider and MacMahon, 1998). Aδ nociceptors can also be functionally subdivided into two categories (Leem et al., 1993; Treede et al., 1995). Type I Aδ nociceptors can be activated by noxious mechanical stimuli or by heat at temperatures higher than 52°C. Type II Aδ nociceptors are also sensitive to both mechanical and heat stimuli but exhibit a lower temperature threshold of 43°C, similar to that of nociceptive C fibers.

Figure 1. Types of nociceptors (Adapted from Basbaum et al., 2009)
2.2.2 Activation of nociceptors

Inflammatory mediators are released at peripheral site during tissue injury which may act via the surface receptors of nociceptors or by internalization, and in turn lead to activation of several ion channels resulting in depolarization of nociceptive neurons. This phenomenon is called inflammatory hyperalgesia. The depolarized nociceptors up-regulate the expression and secretion of inflammatory neuropeptides like SP and CGRP by DRG. These neuropeptides are distributed to both ends of nociceptors through peptidergic fibers. Antidromic transport of neuropeptides to peripheral site further helps in sustained inflammation. This phenomenon is called neurogenic inflammation. On the other hand, transported inflammatory mediators reaching to dorsal horn of spinal cord stimulate post synaptic spinal neurons as well as neighboring glial cells. Inflammatory mediators act as messengers to develop a cross talk between neurons and glial cells, immunocompetent cells, sympathetic terminals, etc. which leads to hypersensitivity of dorsal horn neuron.

![Figure 2. Activation of nociceptors by inflammatory mediators](image-url)

(Dotted arrows show transport of inflammatory mediators)
2.3 Peripheral and central sensitization
The peripheral terminals of primary sensory neurons, when exposed to inflammatory mediators of damaged tissue, lead to reduction in threshold leading to hypersensitivity of nociceptors. Thus amplification in the responsiveness of nociceptors contributes to pain hypersensitivity at inflamed sites. This is known as peripheral sensitization. It generally requires ongoing peripheral pathology for its maintenance (Hucho et al., 2007; Guenther et al., 2001).

Central sensitization is caused due to nociceptive hypersensitivity long after the disappearance of initial injury. During central sensitization, dorsal horn neurons are hypersensitized by a complex phenomenon. Apart from inflammatory signals it may be achieved by several different cellular processes including increase in membrane excitability, a facilitation of synaptic strength and decrease in inhibitory transmitters like GABA (Latremoliere and Woolf, 2009). The molecular mechanism may involve different molecular effectors including kinases like PKA, PKC, CaMKII, and ERK1/2. These kinases participate in changing the threshold and activation of NMDA and AMPA receptors and in their trafficking to the membrane (Slack et al., 2004; Slack et al., 2005; Lee et al., 2009).

2.4 Hyperalgesia and Allodynia
When mild painful stimuli elicit pain of greater intensity as a result of activation of nociceptors, it is referred to as hyperalgesia; whereas normally innocuous stimuli such as light touch or warmth are perceived as painful feeling in case of allodynia. A single mechanism (i.e. sensitization of nociceptive nerve endings) leading to a shift in the stimulus-response function to lower stimulus intensities always causes allodynia and hyperalgesia in combination.
2.4.1 Primary and secondary hyperalgesia
Peripheral stimulation of nociceptors at the site of injury produces sharp intense pain, called primary hyperalgesia. The activation of nociceptors leads to the release of neuropeptides from their terminals. These neuropeptides cause vasodilation. The release of neuropeptides is not restricted to the site of injury. It is also distributed to other terminals of nociceptors outside the area of injury via horizontal transmission, leading to slow and diffused pain in that area. It is known as secondary hyperalgesia.

2.4.2 Acute and Chronic hyperalgesia
A sharp and well-defined pain immediately after tissue injury is categorized as acute hyperalgesia. It acts as a warning system to detect noxious stimuli and to avoid potential tissue damage. Noxious stimulation of peripheral tissue leads to hypersensitivity of specialized peripheral nerves (nociceptors) causing acute hyperalgesia. In case of an injury, inflammatory mediators produced by damaged tissue act on the peripheral terminals to produce sharp and short lived pain. Local tissue inflammation helps the healing process while acute pain protects a damaged body part while it heals. It goes away when there is no longer underlying cause for the pain. The individuals incapable of detecting painful stimuli such as piercing pain from a sharp object, heat, or discomfort associated with internal injuries do not engage appropriate protective behaviors against these conditions, which may be life threatening.
Chronic hyperalgesia continues even after the injury has healed. It includes sensitization of central nerve terminals in spinal cord and brain. A number of downstream genes are proposed to be activated during transition from acute to chronic pain (Basbaum et al., 2009). Like acute pain, it is also associated with local tissue inflammation and alterations of the pain pathway which lead to hypersensitivity. This long lasting pain outlives its usefulness as an acute warning system and becomes chronic and debilitating. Usefulness of chronic hyperalgesia could not be justified till date, therefore it is considered as a pathological condition. Such chronic neuropathy is associated with a number of diseases. Individuals suffering from arthritis, post herpetic neuralgia, or bone cancer experience intense and often unremitting pain that is not only physiologically and psychologically devastating, but may also hamper recovery.

Chronic hyperalgesia is a major clinical problem which could not be fully addressed till date. Presently, non steroidal anti-inflammatory drugs (NSAID) are effective in treating acute inflammatory pain, although with serious side effects. Recently COX-2 specific inhibitors have replaced nonspecific COX inhibitors to reduce side effects. However, COX-2 inhibitors also cause severe side effects like increased risk of myocardial infarction and stroke. On the other hand, opioids offer the most effective treatment for acute and chronic severe pain. However, their clinical utility is limited by the development of opioid induced hyperalgesia in due course of treatment (Ossipov et al., 2004). Steroids are also used to treat inflammatory pain, however they have non-specific targets. Thus the limitations of available modern treatment options have motivated scientists to develop new approaches for treatment of pain.

### 2.5 Role of inflammation in hyperalgesia

Tissue damage or injury is accompanied by concomitant release of inflammatory mediators from local resident cells (endothelial cells, keratinocytes, and fibroblasts), infiltrated cells (mast cells, basophils, platelets, macrophages, neutrophils) and from activated nociceptors. These mediators collectively constitute the inflammatory soup, which consists of wide range of signaling molecules including eicosinoids, cytokines, chemokines, neuropeptides, neurotrophins, as well as extracellular proteases and protons. These pro-inflammatory agents act through the one or more cell surface receptors
expressed on nociceptors. As a final outcome, excitability of the nerve fibers is enhanced; thereby sensitivity to temperature or touch is heightened.

**Figure 4.** Pro-inflammatory mediators involved in hyperalgesia
(Adapted from Meyer et al., 2008)

### 2.5.1 Pro-inflammatory cytokines and hyperalgesia

Pain modulation by pro-inflammatory cytokines has been studied in several animal models showing that tumor necrosis factor-alpha (TNF-α), interleukin (IL-1β), and IL-6 induce and maintain hyperalgesia. Injury of peripheral nervous tissue leads to a rapid and sustained increase in cytokine secretion leading to pain behavior (Üçeyler et al. 2007; Wang et al., 2015; Huang et al., 2017).

#### 2.5.1.1 Tumor necrosis factor-α (TNF-α)

Inflammatory cytokine TNF-α is known to play a well established key role in several pain models (Wu et al., 2014; Ferraz et al., 2015; Carvalho et al., 2015; Huang et al., 2017). TNF-α modulates both inflammatory and neuropathic hyperalgesia by initiating a cascade of inflammatory cytokines; therefore its inhibitors show significant anti-hyperalgesic effects (Chen et al., 2012; Lima et al., 2014; Nascimento et al., 2015; Yang et al., 2017). TNF-α receptors are expressed in both neurons and glial cells (Boka et al., 1997). TNF-α acts on different signaling pathways through cell surface receptors TNFR1
and TNFR2 to activate stress-activated protein kinases (SAPKs) and NF-kB during inflammation, which further activate cascade of other cytokines, notably IL-1β, IL-6 and IL-8 in the inflammatory models of carrageenan induced and zymosan induced hyperalgesic rats (Xu et al., 2017; Ohishi S, 2000). TNF-α activates TTX-R Na+ channels via p38 MAPK pathway in cultured DRG cells (Jin and Gereau, 2006). Literature suggests that TNF-α mediates central mechanisms of neuropathic pain through glial systems (Gruber-Schoffnegger et al., 2013). In response to nerve injury and inflammation, microglia secrete pro-inflammatory cytokines including TNF-α (Ren and Dubner, 2008; Vilhardt F, 2005), which mediate its effects via the p38-MAPK pathway (Schafers et al., 2003). TNF-α auto-stimulates its own production via G-protein coupled receptor (CXCR4) and TNF-α converting enzyme (Watkins and Maier, 2003; Watkins et al., 2007).

Despite the significant role of TNF-α in neuropathic as well as inflammatory pain, the failure of TNF-α antagonists in clinical trials guided research towards a collective role for glia-derived different mediators and their signaling pathways in the modulation of hyperalgesia (Korhonen et al., 2006; Cohen et al., 2009).

2.5.1.2 Interleukin-1β (IL-1β)

IL-1β is primarily released by monocytes, macrophages, fibroblasts and endothelial cells during cell injury and inflammation. It is also reported to be expressed in nociceptive DRG neurons and spinal cord (Crapy et al., 2001; Piccinelli et al., 2017). It is known to play a key role in several pain models (Gruber-Schoffnegger et al., 2013; Ferraz et al., 2015; Wang et al., 2015; Carvalho et al., 2015). IL-1β signals through complex signaling cascades that lead to the release of other nociceptive molecules such as PGE2, IL-6, SP and MMP9 in a number of neuronal and glial cells (Samad et al., 2001; Economides et al., 2003; Kawasaki et al., 2008). Additionally, IL-1β has been shown to cause an increase in the heat-evoked release of CGRP from rat cutaneous nociceptors in vitro (Opree and Kress, 2000). RT-PCR and in situ hybridization studies have demonstrated expression of IL-1R1 in sensory neurons (Crapy et al., 2001; Obreja et al., 2002), which suggests that IL-1β may directly acts on nociceptors. Administration of IL-1ra is reported to reduce CFA-induced upregulation of nerve growth factor (NGF), a neurotrophic factor known to play a crucial role in a variety of acute and chronic pain states (Safieh-
Garabedian et al., 1995). Upregulation of NGF by IL-1β is known at both the transcriptional and post-transcriptional levels (Vige et al., 1991). IL-1β is known to modulate neuronal excitability by affecting neuronal receptors such as TRPV1, sodium channels, GABA receptors and NMDA receptors (Schäfers and Sorkin, 2008).

2.5.1.3 Interleukin-6 (IL-6)

IL-6 contributes to the development of inflammatory and neuropathic pain after a peripheral nerve injury (Dubovy et al., 2013; Zhou et al., 2016; Huang et al., 2017), for example sciatic cryoneurolysis involving repeatedly freezing and thawing a section of the sciatic nerve, results in increased IL-6 expression in the spinal cord (DeLeo et al., 1996). IL-6 is secreted by activated microglia and astrocytes, and regulates neuropeptide expression in neurons (Klein et al., 1997). In addition, intrathecal injection of IL-6 induces tactile allodynia and thermal hyperalgesia in intact and nerve-injured rats (Zhou et al., 2016).

PGE2 up-regulates expression of IL-6 via EP4 receptor, and activates PKC pathway in injured nerves in case of neuropathic pain model (Ma et al., 2005). The role of PGE2 is also demonstrated in the synthesis of IL-6 in primary sensory neurons following nerve injury. In vitro studies suggest that EP4 receptor, PKA, PKC, ERK/MAPK, CREB, and NF-kB signaling pathways are involved in PGE2-induced IL-6 production in DRG neurons (St-Jacques et al., 2011). IL-6 mainly activates the JAK/STAT transduction pathway in microglia of spinal cord during neuropathic pain (Dominguez et al., 2008). There is evidence of IL-6 induced microglial CX3CR1 expression in the spinal cord through p38 MAPK activation after peripheral nerve injury (Lee et al., 2010).

2.5.2 Role of inflammatory enzymes in hyperalgesia

COX-2 and iNOS are the two most important inflammatory enzymes that are implicated in inflammatory hyperalgesia. There are important and complex interactions between these two mediator systems.

2.5.2.1 Inducible nitric oxide synthase (iNOS)

Over activation of different isoforms of nitric oxide synthase plays important role in hyperalgesia by mediating neuronal excito-toxicity by activating NMDA receptors and downstream signaling pathways via p38, JNK, and ERK. Major contributors to neuronal toxicity and hyperalgesia are nNOS and iNOS.
iNOS is found to be expressed in immune cells including glial cells, and is involved in several signaling pathways of hyperalgesia (Levy and Zochodne, 2004). During hyperalgesia, iNOS expression in astrocytes and microglia is found significantly greater than that in neurons (Saha and Pahan, 2006; Sun et al., 2015). Pro-inflammatory cytokines like TNF-α, IL-1β and interferon-γ induce the expression of iNOS in microglia (Kakita et al., 2013). These extracellular mediators lead to up regulation of iNOS expression by activation of NF-κB causing peroxynitrite injury in peripheral nerve (Dogonay et al., 2002). Nitrosative stress from iNOS in axons and Schwann cells, rather than DRG neurons plays a major role in peripheral nerve dysfunction and degeneration (Pavlov and Obrosova, 2008).

2.5.2.2 Cyclooxygenase-2 (COX-2)

COX plays a key role in biosynthesis of prostaglandins from arachidonic acid. Prostaglandins are important for a large number of normal physiological processes including reproduction, immune responses, protection of gastrointestinal mucosa, maintenance of renal homeostasis and regulation of blood clotting etc. Prostaglandins have implication in promoting inflammation and hyperalgesia. Pro-inflammatory cytokines like TNF-α induces the expression of COX in cultured DRG neurons (Fehrenbacher et al., 2006). In addition, prostaglandins seem to be involved in the TNF-α induced sensitization of the TRPV1 receptor but not in its expression (Nicol et al., 1997).

There are two isoforms of cyclooxygenases; COX-1, which is constitutively expressed and COX-2, an inducible COX which is expressed in inflammatory cells and tissues in response to cellular activation by cytokines, mitogens and other stimuli (Smith et al., 2000). However, recently COX-2 is shown to be expressed constitutively in human kidney and central nervous system. COX-2 immunoreactivity has been observed in renal vasculature, medullary interstitial cells and macula densa (Kirkby et al., 2016). Hyperalgesia is developed after inflammation due to increase in PGE2 level in the inflamed tissue and in spinal cord, and is associated with induced COX-2 (Ibuki et al., 2003; Guay et al., 2004). PGE2 is under regulation of COX-2 in case of inflammatory pain; consequently COX-2 selective inhibitors are potent antihyperalgesic agents. COX-2 specific inhibitor coxibs markedly reduce pain symptoms in rat models of carrageenan, zymosan or formalin-evoked hyperalgesia (Niederberger et al., 2001; Veiga et al., 2004;
Svensson et al., 2002; Popp et al., 2009). In contrast to well accepted role of COX-2 in secondary hyperalgesia, its role in primary hyperalgesia is disputed and inconsistent reports show species specific variations in rodents. Selective inhibition of COX-2 is reported to reverse carrageenan mediated primary hyperalgesia in rat but not in zymosan and carrageenan induced mice (Mazario et al., 2001; Jain et al., 2008). COX-2 plays an important role in central sensitization after peripheral inflammation in the mouse and rat models of inflammation (Seybold et al., 2003; Ghilardi et al., 2004). Recent findings show the activation of both COX-1 and COX-2 in the DRG during inflammatory hyperalgesia leading to activation of TRPV1 ion channels as well as PKCε (Araldi et al., 2012). Subsequent release of prostaglandins in the DRG activates and sensitizes primary afferent neurons via autocrine signaling mechanism.

2.5.3 TNF receptor 1 (TNFR1)

There are two types of TNF receptors namely 55 kDa TNFR1 and 75 kDa TNFR2, and both are localized in DRG neurons (Shubayev and Myers, 2001; Pollock et al., 2002; Schäfers et al., 2003). TNF-α signaling during hyperalgesia is mediated mostly via TNFR1 (Yamacita-Borin et al., 2015), whereas both receptors are reported to be involved in cancer induced pain (Gies et al., 2010). Inglis et al. (2005) and Li et al. (2004) have identified TNFR2 only in non-neuronal cells of DRG. The administration of soluble TNFR1 is reported to attenuate mechanical allodynia in a rat model of neuropathic pain (Parada et al., 2003; Sommer et al., 1998). Similarly, intrathecal injection of neutralizing antibodies against TNFR1 in mice subjected to chronic constriction injury (CCI) is shown to reduce thermal hyperalgesia and mechanical allodynia, while application of neutralizing antibodies against TNFR2 did not (Sommer et al., 1998). In addition, antisense oligo-dNTPs against TNFR1 reduced hyperalgesia (Parada et al., 2003). TNF-α caused an increase in TTX-R Na+ currents in DRG neurons from tnfr2−/− mice but not from tnfr1−/− mice (Jin and Gereau, 2006). Induction of monocyte chemoattractant protein-1 (MCP-1) by tumor TNFR1 in sensory neurons contributes to induction of neuropathic pain in the ventral rhizotomy model (Jeon et al., 2011). These findings further support the importance of TNFR1 in nociception.
2.6 Neuronal marker of pain: c-Fos

The expression of c-Fos is rapidly and selectively up-regulated in response to a wide variety of cellular stimuli, and therefore is known as immediate early gene (Greenberg et al., 1986). c-Fos protein forms a complex with Jun, which binds to the AP-1 response element to induce gene transcription. Hunt et al. (1987) have shown dramatic increase of c-Fos expression in spinal dorsal horn following noxious stimuli; and its pattern was topographically correlated with primary afferent projection from the hind paw. Since then, c-Fos has been widely used as a neural marker of nociception and for the testing the efficacy of analgesic compounds (Coggeshall RE, 2005; Yang et al., 2010; Jawed et al., 2015; Uchytilova et al., 2015). c-Fos is expressed only in nuclei of neurons, and therefore c-Fos immunohistochemistry is performed in combination with tract-tracing procedures to identify the properties of c-Fos labeled neurons. Correlation between c-Fos expression and the stimulus intensity has been widely investigated; however role of c-Fos in pain regulation remains to be established. Subcutaneous injection of chemicals, such as capsaicin, carrageenan, and formalin induces dose-dependent c-Fos expression in the spinal dorsal horn (Hossaini et al., 2014; Jinks, 2002). Fos-IR neurons are mostly found in laminae I-II, with a few in laminae III-IV and some in laminae V-VI (Coggeshall et al., 2005; Hossaini et al., 2014). As stimulus intensity increases, not only the number of c-Fos-IR neurons increases, but also the territories expressing c-Fos expand from superficial to the deep dorsal horn (Hossaini et al., 2014; Hsieh et al., 2015). As an immediate early gene, c-fos mRNA can be activated within minutes of stimulation, with a peak accumulation in 30 minutes (Molander et al., 1994). But the expression of c-Fos protein is significantly delayed. After an acute noxious stimulation such as formalin injection in hind paw, c-Fos protein expression begins at 30 minutes, peaks at 1-2 h and returns to basal levels after 8-24 h (Harris JA, 1998).

Despite the well accepted marker of pain, the mechanism underlying c-Fos-mediated pain regulation is largely unknown. Experiments performed to detect its role in pain behavior resulted in inconsistent findings. Several studies showed that c-fos anti-sense oligo-dNTPs decrease formalin-induced nociceptive behavior, bee venom-induced nociceptive behavior and adjuvant-induced thermal hyperalgesia (Hou et al., 1997; Sugio et al., 2001;
Wu et al., 2002). On the contrary, Hunter et al. (1995) and Ibrahim et al. (2001) have reported that c-fos anti-sense oligo-dNTPs increase formalin-induced pain.

2.7 Reactive oxygen species (ROS)

ROS includes a number of reactive molecules and free radicals derived from molecular oxygen. ROS has the potential to cause a number of deleterious events by oxidation of macromolecules. ROS is produced as by-products during the mitochondrial electron transport of aerobic respiration, or by oxidoreductase enzymes and metal catalyzed oxidation. ROS has a role in cell physiology including apoptosis, as well as in immune response. A balance in total ROS of an organism is important for homeostasis. Production of ROS is balanced under normal physiological conditions, by several cellular antioxidant mechanisms (Jenner P, 1994).

The imbalance between production of ROS and biological system's ability to readily detoxify the reactive intermediates leading to accumulation of ROS is described as oxidative stress. Oxidative stress may arise from exogenous origins like ultraviolet rays and/or endogenous origins at the cellular level where mitochondria are involved. Oxidative stress is closely related to many of the pathological conditions.

Apart from the general oxidative role, ROS is shown to have specific targets by modulation of gene expression and activation of corresponding signaling cascades. Recent studies have shown the involvement of ROS in hyperalgesia. These are implicated in neuropathic as well as inflammatory pain (Lochhead et al., 2012; Xu et al., 2014). ROS is known to mediate development and maintenance of capsaicin-induced hyperalgesia in mice, primarily through central sensitization (Lee et al., 2007; Schwartz et al., 2008). Free radical scavengers, such as phenyl N-tert-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) produce analgesic effects in neuropathic and inflammatory pain (Wang et al., 2004; Kim et al., 2004; Yowtak et al., 2011; Maixner et al., 2016). However, identification of molecular mechanism of anti-hyperalgesic effect of antioxidants is necessary to use them as therapeutic drug in hyperalgesia, which is not yet established due to limited studies available regarding molecular targets of antioxidants.
2.8 Oxidative stress induced injury

Oxidative stress due to accumulation of excessive ROS leads to oxidation of DNA, lipids (including phospholipids), proteins and carbohydrates, which hampers their functional abilities. Degree of oxidative stress in the local environment modulates the cellular physiology via various signaling pathways.

2.8.1 Lipid peroxidation

Lipid peroxidation leads to cellular instability due to membrane modification. This is considered as the foremost reason of cellular injury and is used as an indicator of oxidative stress in cells and tissues. The oxidative destruction of polyunsaturated fatty acids (PUFAs), mostly present in cell membrane is known as lipid peroxidation. Regulated phenomena of lipid peroxidation offered a proper balance between the actions of some enzymatic and non-enzymatic systems that are opposed to the generation of ROS by mitochondrial respiration (Kuhn et al., 2002). The enzymatic system includes pro-oxidative enzymes as well as reducing enzymes like glutathione peroxidases (Cejas et al., 2004). The initial reaction with PUFAs produces a lipid radical which abstracts hydrogen atom from the neighboring fatty acids to produce lipid hydroperoxides (LOOH) and a second lipid radical. The LOOH undergoes reductive cleavage by reducing metals and produces alkoxyl radical. Both alkoxyl and peroxyl radicals create a chain reaction by abstracting additional hydrogen atoms. Lipid peroxidation is one of the most investigated consequences of ROS actions on membrane structure and function.

2.9 Antioxidant defense system

ROS like superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (HO') are constantly generated in vivo during normal cellular processes and metabolism. Cell possesses powerful exogenous and endogenous defense system to protect against the potentially damaging effects of ROS. Endogenous antioxidants are enzymatic and non-enzymatic in action (Mates et al., 2008). Exogenous antioxidants may be natural antioxidants flavonoides, vitamins and synthetic including butylated hydroxyanisol (BHA) and butylated hydroxyl toluene (BHT). The enzymatic endogenous antioxidants are catalase, superoxide dismutase and glutathione peroxidase and non-enzymatic antioxidants are thiol, glutathione, ascorbate, urate etc which protect against oxidative stress. Both enzymatic and non-enzymatic antioxidants contribute to the body’s major
defense system against free radicals. They react with free radicals and provide protection against the toxic effect of ROS by chelating or scavenging them. Hence, antioxidant status has been suggested as a useful tool in estimating risk of oxidative damage induced hyperalgesia.

2. 9.1 Antioxidant enzymes

Endogenous primary antioxidant enzymes are catalase, superoxide dismutase and glutathione peroxidase. In addition glutathione reductase, glutathione s-transferase and NAD(P)H: quinine oxidoreductase 1 are described as phase-II secondary antioxidant enzymes.

2. 9.1.1 Catalase (CAT)

Catalase (EC. 1.11.1.6) is an intracellular antioxidant enzyme present in almost all the mammalian cells. It is located in cellular peroxisomes and to some extent in cytosol. It is a 240 kDa hemoprotein containing enzyme having four ferriprotoporphyrin groups per molecules. It catalyzes decomposition of \( \text{H}_2\text{O}_2 \) to water and molecular oxygen and oxidation of \( \text{H} \) donors (methanol, ethanol, formic acid and phenol (Young and Woodside, 2001)).

\[
\begin{align*}
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2 \\
\text{ROOH} + \text{AH}_2 \xrightarrow{\text{Catalase}} \text{H}_2\text{O} + \text{ROH} + \text{A}
\end{align*}
\]

Catalase is one of the most efficient enzymes having peroxidase activity which reacts with organic peroxide to protect the cells from oxidative stress. Superoxide radicals inhibit catalase activity (Quan et al., 2011). The level of catalase shows great heterogeneity in different tissues of mammalian system. The level is high in liver, erythrocytes and low in brain, skeletal muscle and pancreas. Catalase is potentially important in metabolism of endogenous substrate and inactivation of carcinogens (Vetrano et al., 2005). Its peroxisomal localization suggests that other defense systems may also be responsible for the removal of \( \text{H}_2\text{O}_2 \). Catalase may jointly fulfill the role of a terminal oxidase, catalyzing the reduction of \( \text{O}_2 \) to water. Catalase has high capacity for reaction but a relatively low affinity for its substrate.

2. 9.1.2 Superoxide dismutase (SOD)

Superoxide dismutase (EC 1.15.1.1) is a prominent scavenger of superoxide radicals (\( \text{O}_2^- \)). It is a family of metalloproteins found in both prokaryotic and eukaryotic cells
which catalyze the spontaneous dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. Hydrogen peroxide is further removed by catalase or glutathione peroxidase (Quan et al., 2011).

\[
O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2
\]

Another function of superoxide dismutase is to protect dehydratases like dihydroxy acid dehydratase, acotinase, 6-phosphogluconate dehydratase and fumarases A and B against inactivation from the free radical superoxide (Benov and Fridovich, 1998). There are three isoforms of superoxide dismutase found in mammalian tissues, each with a specific sub cellular location and different tissue distribution. Manganese superoxide dismutase (MnSOD) removes \(O_2^-\) generated by one electron leakage from the electron transport chain. Copper Zinc superoxide dismutase (CuZn-SOD) is a dimeric protein, found in the cytoplasm where it removes \(O_2^-\) generated by endoplasmic reticulum (Chan et al., 2006). The third SOD isoform is extracellular superoxide dismutase (EcSOD), found in extracellular space and removes membrane associated oxidase generated super oxide radicals (Macmillan and Cruthirds, 2001).

### 2. 9.1.2 Manganese superoxide dismutase (Mn-SOD)

Mn-SOD is localized in the mitochondrial matrix of almost all cells. It is a homotetramer (88kDa) containing one manganese atom per subunit that cycle from Mn (III)-Mn (II) and back to Mn (III) during two step dismutation of superoxide. The respiratory chain in mitochondria is the major source of oxygen radicals. Mn-SOD is a primary antioxidant enzyme that functions to remove these superoxide radicals. The amino acid sequence of Mn-SOD is entirely dissimilar to that of CuZn-SOD and it is not inhibited by cyanide, allowing Mn-SOD activity to be distinguished from that of CuZn-SOD in mixtures of the two enzymes. The expression of Mn-SOD is essential for the survival of aerobic life and the development of cellular resistance to oxygen radical mediated toxicity (Zhang et al., 2002).

### 2. 9.1.2.2 Copper-Zinc superoxide dismutase (CuZn-SOD)

CuZn-SOD is conserved throughout evolution which usually has two identical subunits of about 32kDa, each containing a metal cluster in the active site constituted by a copper and a Zinc atom bridged by a common ligand His 61. Inactivation of copper and Zinc containing SOD by \(H_2O_2\) is the consequence of several sequential reactions. First,
reduction of the active site Cu(II) to Cu(I) by H₂O₂, then oxidation of the Cu(I) by a second H₂O₂, thus generating a powerful oxidant which may be Cu(I)O, Cu(II)OH or Cu(III) and finally oxidation of the histidine causes loss of SOD activity. CuZn-SOD plays a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen (Zhang et al., 2002).

2. 9.1.3 Glutathione Peroxidase (GPx)

Glutathione peroxidase (EC 1.11.1.19) is a selenium containing antioxidant enzyme, which catalyzes the reduction of hydroperoxides (ROOH and H₂O₂) using GSH, thereby protecting mammalian cells against oxidative damage.

\[
\begin{align*}
\text{ROOH} + 2\text{GSH} & \xrightarrow{\text{GPX}} \text{ROH} + \text{GSSG} + \text{H}_2\text{O} \\
\text{H}_2\text{O}_2 + 2\text{GSH} & \xrightarrow{\text{GPX}} 2\text{H}_2\text{O} + \text{GSSG}
\end{align*}
\]

There are five isozymes of glutathione peroxidase (GPX) found in mammals. Although the expression of GPx is ubiquitous, the level of each isoform varies depending up on tissue type. Glutathione peroxidase1 (GPX1) reduces fatty acid hydroperoxides and H₂O₂ at the expense of glutathione (Arsova-Sarafinovska et al., 2009). GPx2 and GPx3 are poorly detected in most tissues except the gastrointestinal tract and kidney (Falck et al., 2010). However GPx4 is highly expressed in renal epithelial cells and testis (Ueta et al., 2011). GPx4 is located in cytosolic and membrane fractions which can directly reduce the phospholipid hydroperoxides, fatty acid hydroperoxides and cholesterol hydroperoxides that are produced in peroxidized membrane and oxidized lipoproteins. GPx5 is expressed specifically in mouse epididymis (Ding et al., 1998). The glutathione redox cycle is a major source of protection against low levels of oxidative stress.

2. 9.1.4 Glutathione reductase (GR)

Glutathione reductase (E.C. 1.6.4.2) is grouped under antioxidant enzymes by its ability to catalyze the reduction of GSSG to GSH and maintaining the level of reduced glutathione. Glutathione reductase is flavine nucleotide dependent enzyme and has a similar tissue distribution to glutathione peroxidase. The activity of glutathione peroxidase is dependent on the constant availability of reduced glutathione. The ratio of reduced to oxidized glutathione is very high in cells due to the activity of glutathione
reductase. The enzyme requires NADPH as a coenzyme for reduction of glutathione disulphide (Kaneko et al., 2001).

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+
\]

### 2.9.2 Role of antioxidant enzymes in hyperalgesia

Hyperalgesic responses are reported to be associated with modulation in activities of antioxidant enzymes; however different studies show variation in the enzyme activities. Naik et al. (2006) have demonstrated a decreased SOD activity and unaltered catalase activity in sciatic nerve in CCI model of neuropathic pain, whereas decreased activity of both catalase and SOD in spinal cord of SNT model of neuropathic pain has been reported by Guedes et al. (2006). In contrast, Varija et al. (2008) have reported increased SOD and GPx activities, and decreased catalase activity in different tissues of silver wire ligated neuropathic pain model. Discrepancies in these reports might be a result of differences in animal models, tissues or time of estimation of enzyme activities. However, these discrepancies in the enzyme activities could not be justified in different models of hyperalgesia. Wang et al. (2004) reported decreased activity of Mn-SOD by nitration in spinal cord during hyperalgesia. More studies are needed to establish a functional relationship between activities of these enzymes and development of hyperalgesia. We have recently reported that antioxidant enzymes mainly modulates initiation of hyperalgesia (Singh and Vinayak, 2015; Singh and Vinayak, 2017).

### 2.10 Role of ROS in hyperalgesia

Increasing body of evidence indicates a crucial role of ROS in pain signaling. Wang et al. (2004) have for the first time identified superoxide (SO) as a mediator of hyperalgesia. They have shown that nitration of spinal Mn-SOD provides a feed forward mechanism that allows the accumulation of SO and peroxinitrate causing its inactivation. Hydrogen peroxide is also identified as a mediator of inflammatory pain (Keeble et al., 2009). ROS may induce peripheral/central sensitization by increasing phosphorylation of AMPA or NMDA receptors or by increased activity of ion channel TRPV1. ROS originated in mitochondria as well as produced by cell membrane associated NADPH oxidase (NOX) are reported to induce hyperalgesia (Ibi et al., 2008; Li et al., 2011). Studies on knock out animals have revealed the role of NOX1 as well as NOX2 isozymes in hyperalgesia (Ibi
et al., 2008; Kim et al., 2010). Ibi et al. (2008) demonstrated the accelerated PKCε translocation in DRG neurons in response to NOX-derived SO production during development of thermal and mechanical hyperalgesia. ROS is important for initiation as well as maintenance of hyperalgesia (Singh and Vinayak, 2017). Involvement of ROS in hyperalgesia may be further supported by alleviation of hyperalgesia after treatment with natural antioxidants like curcumin (Yeon et al., 2010), resveratrol (Tao et al., 2016; Wu et al., 2017) and 6-gingerol (Young et al., 2005) etc.

2.11 Alteration of cellular signaling by ROS

ROS is implicated in the pathogenesis of a wide variety of human diseases such as cancer and cardiovascular diseases. Excessive ROS level may inflict direct damage to vital cell constituents such as lipids, proteins and DNA. ROS is implicated in normal physiological processes. It activates a number of signaling proteins and enzymes which regulate cell growth, differentiation and apoptosis (Mates et al., 2008). ROS acts as secondary messenger responsible for a signal transduction from extracellular signaling molecules and their membrane receptors to the intracellular regulatory systems which control gene expression. The transcriptional regulation by ROS is mediated mainly via activation of MAP kinases and transcription factors like AP-1 and NF-kB. ROS-mediated gene regulation, modulated under oxidative stress leads to ROS associated disorders (Weigel et al., 2002). A large number of genes are regulated in cells treated with H2O2 (Hensley et al., 2000).

2.11.1 Src family kinase (SFK)

Src family kinase (SFK) represents a family of non-receptor tyrosine kinases, including nine members: Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn and Frk. SFKs are widely expressed in the mammalian nervous system and have been implicated in the development of CNS (Hoffman-Kim et al., 2002). Increasing body of evidence indicates that SFKs act as a point of convergence for various signaling pathways and may play a crucial role in the processes underlying physiological plasticity including learning and memory; and pathological plasticity including epilepsy and pain (Purcell and Carew, 2003; Salter and Kalia, 2004). Role of SFK in development of hyperalgesia is well reported (Katsura et al., 2006; Tan et al., 2012; De Felice et al., 2016). Src is expressed in the DRG as well as in the spinal cord and contributes to the development of
inflammatory pain hypersensitivity (Igwe OJ, 2003; Guo et al., 2004; Igwe OJ, 2013). SFK mediated ERK activation contributes to mechanical hypersensitivity after nerve injury (Katsura et al., 2006). Src inhibition in spinal dorsal horn leads to attenuation of inflammatory pain by down regulating the function of NMDARs (Suo et al., 2013, Lai et al., 2016). ROS induced activation of SFK is evidenced by several line of evidences for example, intracellular ROS activates Src during cell adhesion and anchorage (Giannoni et al., 2005), however involvement of SFK in ROS mediated hyperalgesia is not studied till date.

2.11.2 Protein tyrosine phosphatase (PTP)

Reversible phosphorylation of proteins is the classical way of signaling by which living organisms coordinate the function of their constituting cells. Relative activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) maintain protein phosphotyrosine level. Super-family of PTPs consists of enzymes which catalyze the dephosphorylation of phosphotyrosine, as well as dual-specificity phosphatases (DUSPs) which remove phosphate groups from phosphothreonine and phosphoserine in addition to tyrosine. Some PTP family members also dephosphorylate phospholipids, phosphorylated carbohydrates or oligonucleotides (Alonso et al., 2004; Tonks et al., 2006, Pulido et al., 2013). Research over the past decade has decisively established the important roles for PTPs in cellular signaling pathways showing its implication in various diseases. PTPs are transiently activated only at appropriate time to relay a specific signaling pathway. However, role of PTPs in hyperalgesia is started to be revealed recently (Suo et al., 2013; Azkona et al., 2016).

All PTPs contain an active-site motif with a conserved cysteine residue which is essential for phosphate recognition and catalysis (Tonks NK, 2006). It has been emphasized that ROS may regulate cellular signaling by reversible oxidation of cysteine residues of PTPs (Klomsiri et al., 2011). Therefore, involvement of PTP may be suggested during ROS induced hyperalgesia.

2.12 MAP kinase (MAPK)

Members of MAPK constitute a family of evolutionally conserved intracellular signaling molecules. This family includes extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK) and a relatively new and less-known member of the family
ERK5 (Johnson and Lapadat, 2002). These signaling cascades transduce a wide range of stimuli into diverse intracellular responses by both transcriptional and non-transcriptional regulation (Widmann et al., 1999; Johnson and Lapadat, 2002). MAPKs are activated by phosphorylation via upstream MKKs/MEKs and MKKKs/MEKKs. Early studies indicated a critical role of ERK in regulating mitosis, proliferation, differentiation, cell survival and neuronal plasticity in the adult (Aredia et al., 2015; Iwamoto et al., 2016; El Gaamouch et al., 2012). JNK and p38 play essential roles in regulating inflammatory responses, neurodegeneration, and cell death (Gao and Ji, 2008; Zhan et al., 2015).

2.12.1 Role of MAPK pathway in hyperalgesia

Other than the classical role of MAPKs in cell death and survival, studies in the last decade revealed direct or indirect involvement of all the three MAPKs in neuropathic and inflammatory hyperalgesia. Although MAPK inhibitors have been shown to alleviate hyperalgesia and allodynia in inflammatory and neuropathic pain models, these inhibitors have little or no effect on basal physiological pain perception (Ji et al., 2007; Ostenfeld et al., 2015), suggesting a specific role of MAPKs in the development of inflammatory or pathological pain. Activation of MAPK in primary sensory nociceptive neurons and neurons of spinal cord dorsal horn (SCDH) plays an important role in peripheral and central sensitization (Ji and Woolf, 2001; Bhave and Gereau, 2004; Obata and Noguchi, 2004). Peripheral or spinal nerve injury activates p38 and ERK in spinal microglia, and JNK in astrocytes (Jin et al., 2003; Tsuda et al., 2004; Zhuang et al., 2005; Hains and Waxman, 2006; Zhuang et al., 2006; Ostenfeld et al., 2013). After nerve injury, ERK activity is reported to increase first in microglia and then in astrocytes in late phase. Recent literature suggests a significant role of p38 in post-operative pain (Alkaitis et al., 2010; Tong et al., 2012; Saha et al., 2013; Lin et al., 2014). Increasing body of evidence indicates a crucial role of glial cells in the pathogenesis of pain (Watkins et al., 2001; Deleo et al., 2004; Ji et al., 2007).

2.12.2 ERK - MAP kinase in hyperalgesia

ERK1 (p44 MAPK) and ERK2 (p42 MAPK) have high homology. Both of them are activated together by upstream kinase MEK1 and MEK2. Furthermore, MEK inhibitors and phosphorylated ERK (pERK) antibodies do not distinguish between ERK1 and ERK2; therefore ERK1/2 is generally referred as a single term “ERK”.
Activation of C-fibers and Aδ-fibers by noxious stimuli leads to ERK activation in spinal cord dorsal horn. Low threshold electrical stimulation (Wang et al., 2004) and tactile stimulation (Hao et al., 2005) also lead to ERK activation in SCDH neurons after nerve injury. A transient noxious stimulation (<10 s) may not be sufficient to activate ERK as it is selectively activated in case of persistent pain sensitivity (Wei et al., 2006). pERK is reported to be induced in SCDH neurons by persistent noxious input, produced by hind paw inflammation with formalin (Karim et al., 2001), complete Freund's adjuvant (Adwanikar et al., 2004; Zhang et al., 2014), scorpion venom (Pang et al., 2008); and by chronic bladder inflammation (Cruz et al., 2005a), monoarthritis in the ankle (Cruz et al., 2005b) and fracture of the femur (Jimenez-Andrade et al., 2007). In addition to SCDH neurons, ERK activation also takes place in primary afferents and brain regions (amygdala neurons) following a peripheral inflammation. Stimulation of DRG neurons with TNF-α leads to ERK activation and subsequent increase in expression of TRPV1 (Hensellek et al., 2007), which is a major target of peripheral sensitization. Further, morphine induced hyperalgesia involves activation of ERK in brain cortex (Sanna et al., 2014). Recent study reveals the role of ERK signaling in the periphery as it influences the transition from acute to chronic postoperative pain (Skopelja-Gardner et al., 2017). Activation of early gene ERK has been recently considered as neuronal marker of pain (Gao and Ji, 2009).

2.13 Exogenous antioxidants

Endogenous antioxidant defense system of an organism is supported with exogenous intake of antioxidants like vitamin C, vitamin A, carotenoids, flavonoids, and polyphenols which further contribute to antioxidant mechanisms. Therefore there is a continuous demand for exogenous antioxidants.

2.13.1 Curcumin

Curcumin is the principal yellow-orange curcuminoid of the popular Indian spice turmeric, which is a common spice and coloring agent, derived from rhizome of Curcuma longa. The curcuminoids are natural phenols that are responsible for yellow color of turmeric. Curcumin can exist in several tautomeric forms, including a 1, 3-di keto form and two equivalent enol forms. The enol form is more energetically stable in solid phase and in solution (Kolev et al. 2005). Curcumin is widely used as a food additive and in
herbal medicine (Singh S, 2007). It has therapeutic effects against many chronic diseases, including neoplastic, neurodegenerative, cardiovascular, pulmonary, and metabolic diseases (Aggarwal and Harikumar, 2009; Aggarwal and Sung, 2009). These medicinal properties could be attributed to its antioxidant and anti-inflammatory effects (Sandur et al., 2007). Curcumin has a vanilloid structure similar to that in capsaicin, which is important for the activation of the TRPV1 receptor (Garle et al., 2000; Martelli et al., 2007). Recent studies demonstrated the anti-hyperalgesic effect of curcumin by antagonism of TRPV1 or by inhibition of CaMKII2α (Yeon et al., 2010; Hu et al., 2016). The antioxidant property of curcumin might also be responsible for anti-hyperalgesic effect. However, the role of antioxidant property of curcumin in hyperalgesia is less studied.

![Curcumin, Vanilloid ring, and Capsaicin](image)

**Figure 5.** Structure of curcumin showing structural similarity with capsaicin

### 2.13.2 Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol and a phytoalexin produced by several plants in response to injury or when the plant is under attack by pathogens such as bacteria or fungi. Sources of resveratrol in food include the skin of grapes, blueberries, raspberries, mulberries. Richest sources of resveratrol are grapes and red wine. It is reported to have anti-aging, antioxidant, anti-carcinogenic and anti-inflammatory properties (Howitz et al., 2003; Kim et al., 2005; Manna et al., 2000; Mattson, 2008; Seo and Kim, 2015; Romano et al., 2013). It has no known toxic side-effects (Russo GL, 2007). Resveratrol reduces the expression of different inflammatory mediators involved in the progression of neuropathological conditions and it has been shown to provide neuronal protection in different models (Anekonda TS, 2006). The anti-
inflammatory activity of resveratrol has been well documented and can be ascribed to the inhibition of pro-inflammatory mediators such as prostaglandins. Pham-Marcou et al. (2008) have shown anti-hyperalgesic effect of resveratrol via inhibition of COX-2. However, literature about detailed mechanism of action of resveratrol is not available, which may involve several mediators besides COX-2, such as pro-inflammatory cytokines, iNOS etc. In fact, a recent report suggests the anti-hyperalgesic effect of resveratrol by maintenance of pro-inflammatory and anti-inflammatory cytokines (Tao et al., 2016). Interestingly, resveratrol may also act as a pro-oxidant in different biological systems (Ahmad et al., 2003). Therefore, further studies are needed to elucidate whether treatment with resveratrol effectively reduces ROS production and if its anti-hyperalgesic effects are mediated via its antioxidant properties.

Figure 6. Structure of resveratrol