Chapter 3:  
*Review of Literature*
REVIEW OF LITERATURE

3.1. PAIN

3.1.1. Definition of Pain: The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience which we primarily associate with tissue damage or describe in terms of such damage, or both (IASP, 1994). According to World Health Organisation (WHO) more than 1/3rd world’s population suffers from persistent or recurrent pain (Sarah et al., 2004; Hoffman et al., 2009). It is a protective mechanism for the body and causes humans or animals to react to remove the pain stimulus. Pain is both a sensation and an emotional experience. Pain is always subjective; and may be affected by emotional, social and spiritual components thus it has been defined as “total pain”. Pain can be acute and chronic in nature. Acute pain is nociceptive in nature, and occurs secondary to chemical, mechanical and thermal stimulation of A–delta and C-polymodal pain receptors. Chronic pain is unrelenting and not self-limiting and as stated earlier, can persist for years and even decades after the initial injury.

3.1.2. The Gate Theory of Pain: Melzack and Wall (1965) proposed ‘gate control” theory of pain and described a synapse in the spinal cord that modulates or “gates” the flow of pain to the brain. They speculated that thin (nociceptive) and large diameter (innocuous) nerve fibers carry information from the site of injury to two destinations in the dorsal horn of the spinal cord: the "inhibitory" cells and the "transmission" cells. Signals from both thin and large diameter fibers excite the transmission cells, and when the output of the transmission cells exceeds a critical level, pain begins. They proposed similarly, that since the gate at the second order neuronal site of the spinal cord could allow pain to flow by “opening,” conversely certain triggers could cause the brain to turn off pain by sending impulses down the spinal cord, and closing the
pain gate (Wall, 1978). This theory led to an important discovery about pain, namely that incoming pain signals are subject to modulation, increasing and decreasing, greatly changing the final perception of its degree by the patient (Romanelli and Esposito, 2004). From a pathophysiological point of view, pain can be classified as nociceptive (somatic and visceral), neuropathic (central, peripheral, sympathetic) idiopathic or psychogenic (Woolf, 2010).

3.1.3. Nociceptive Pain: Nociceptive pain is presumed to occur as a result of the normal activation of the sensory system by noxious stimuli, a process that involves transduction, transmission, modulation and perception (Dubin and Patapoutian, 2010). Nociceptive pain is caused by stimulation of peripheral nerve fibers that respond only to stimuli approaching or exceeding harmful intensity (nociceptors), and may be classified according to the mode of noxious stimulation; the most common categories being "thermal" (heat or cold), "mechanical" (crushing, tearing, etc.) and "chemical" (iodine in a cut, chili powder in the eyes).

Nociceptive pain is mediated by receptors on A–delta and C–fibers which are located in skin, bone, connective tissue, muscle and viscera (Basbaum et al., 2009). It can be somatic or visceral in nature. Somatic pain tends to be well localized, constant pain that is described as sharp, aching, throbbing, or gnawing. Visceral pain, on the other hand, originates in the viscera (organs) and often is extremely difficult to locate, paroxysmal in nature and is usually described as deep, aching, squeezing and colicky in nature (Giamberardino, 1999). Tissue injury activates primary afferent neurons called nociceptors, which are small diameter afferent neurons (with A-delta and C-fibers) that respond to noxious stimuli and are found in skin, muscle, joints, and some visceral tissues. These fibers have specific receptors that may be responsible for noxious mechanical, chemical or thermal stimuli (Dubin and Patapoutian, 2010).
Among the sensory channels TRP channels, acid sensing ion channels (ASICs), bradykinin (B1, B2), and prostaglandin (EP1, EP2) receptors play a key role in sensitization and maintenance of pain (Segond et al., 2003; Bujalska et al., 2009). Also axonal channels such as voltage gated sodium channels, K$^+$ channels and Ca$^{2+}$ channels can contribute to nociceptor activation as they contribute to setting of the membrane potential and modulate discharge behavior (Gover et al., 2009; Su et al., 2010).

Recently receptors for neuropeptides have also been identified in primary afferent neuron, including substance P (neurokinin 1 receptors), and calcitonin gene-related peptide (CGRP receptors) (Cridland et al., 1998; Sun et al., 2003). Interestingly, receptors for inhibitory peptides are also expressed, e.g. receptors for opioids, somatostatin and NPY (Karagiannis et al., 2009). Most of these receptors could be auto-receptors because the neurons with the receptors also synthesize the corresponding neuropeptide. It has been proposed that the activity or threshold of a neuron results from the balance between excitatory and inhibitory compounds. It is conceivable that changes in the expression of ion channels and receptors may contribute to the maintenance of chronic pain (Han et al., 2005). Some of the changes seem to be stimulated by neurotrophins such as nerve growth factor (NGF). Neurotrophins are survival factors during the development of the nervous system, but during inflammation of the tissue, the level of NGF is substantially enhanced (Hellweg et al., 1994). By acting on the tyrosine kinase A (trk A) receptors, NGF increases the synthesis of substance P and CGRP in the primary afferents (Amann et al., 1996). NGF may also act on mast cells and thereby activate and sensitize sensory endings by mast cell degranulation (Lewin et al., 1994). However, the inflammatory mediator
PGE\textsubscript{2} is able to cause an up-regulation of expression of neurokinin 1 receptor in DRG neurons (Segond et al., 2003)

### 3.1.4. Neuropathic Pain:

Nociceptive and neuropathic pain is caused by different neuro physiological processes, and therefore tend to respond to different treatment modalities. Neuropathic pain, in contrast to nociceptive pain, has been considered as the most debilitating painful condition and is described as "burning", "electric", "tingling", and "shooting" in nature (Treede et al., 2008). Neuropathic pain is produced by damage to, or pathological changes in the peripheral or central nervous systems and is characterized by the sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia), and pain in response to a stimulus that does not normally provoke pain (allodynia) (Ueda, 2006).

The mechanisms involved in neuropathic pain are complex and it has been reported that both peripheral and central pathophysiologic mechanism are involved in the genesis of neuropathic pain (Woolf et al., 1992; Tatsuro and Micho, 2006). Peripheral sensitization, a reduction in the threshold of nociceptor afferent peripheral terminals, is a result of inflammation at the site of surgical trauma (Okamoto et al., 2001). Central sensitization, an activity-dependent increase in the excitability of spinal neurons, is a result of persistent exposure to nociceptive afferent input from the peripheral neurons (Woolf et al., 1992; Latremoliere and Woolf, 2009). Taken together, these two processes contribute to the postoperative hypersensitivity state (spinal windup) that is responsible for a decrease in the pain threshold, both at the site of injury (primary hyperalgesia) and in the surrounding uninjured tissue (secondary hyperalgesia) (Cervero et al., 2003).
The sensation of pain results from the firing of specialized first order neurons called nociceptors that transmit signals carried into the central nervous to nociceptive cells, forming connections to second order spinal neurons, eventually leading to the brain (Heinricher et al., 2009; Latremoliere and Woolf, 2009). Once reached by the brain, third order neurons originating in the thalamus transmit the signals into the cerebral cortex, thus the sensation of pain. Moreover, the spinal cord is influenced by descending tracts that reduce or facilitate the nociceptive processing at the spinal level. These pathways originate from brainstem nuclei (in particular the periaqueductal grey, nucleus raphe magnus) and descend in the dorsolateral funiculus of the spinal cord (Hucho and Levine, 2007). These descending pathways and segmental inhibitory neurons provide significant control over the nociceptive processing (Heinricher et al., 2009). However, ischemia, nerve injury and inflammation enhance neuronal sensitivity, resulting hyperalgesia and allodynia via central sensitization (Latremoliere and Woolf, 2009).

3.1.5. Molecular mechanisms of spinal sensitization

Peripheral and central sensitization is induced and maintained by the action of several endogenous mediators, receptor and transmitter systems. A major role is played by excitatory neurotransmitters, including glutamate and aspartate, the main transmitters in nociceptors (Larsson and Broman, 2010; Osikowicz et al., 2008). Glutamate excites postsynaptic neurons by activating ionotropic receptors in the sub synaptic membrane. Importantly, glutamate has also the potential to induce hyper-excitability by activating ionotropic N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors in spinal cord neurons (Li et al., 2010). When NMDA receptors are opened by glutamate, large amounts of calcium flow into the neuron (Huber et al., 1995) and induce second
messenger cascades that increase neuronal excitability (Gover et al., 2009). Administration of MK-801, an antagonist of the NMDA receptor can prevent central sensitization, and established hyper excitability following nerve injury (Chen et al., 2009).

Activation of NMDA receptor is associated with up-regulation of COX expression in the spinal dorsal horn during nociceptive inputs in rats (Li et al., 2009). Further, it has been shown that neuropeptides and spinal prostaglandins are also involved in the process of peripheral and central sensitization (Intondi et al., 2008). Many neurons in the spinal cord express receptors for the tachykinins substance P, neurokinin A, and CGRP (Cridland and Henry, 1998; Sun et al., 2003; Han et al., 2005). During acute inflammation, the spinal release of substance P, neurokinin A and CRGP from nociceptors is increased and these neuropeptides support the generation of spinal cord hyper-excitability (Han et al., 2005). Spinal application of antagonists to these receptors attenuates the development of neuronal injury and inflammation-mediated hyper-excitability (Kawamura et al., 1989; Dionne et al., 1998).

Although the clinical features of inflammatory and neuropathic pain differ substantially, recent data suggest that local inflammation of the peripheral nerves is a crucial part of the generation of neuropathic pain (Kohno et al., 2003). Moreover, non-neuronal cells including glial cells have been shown to be active players in the process of neuronal sensitization and activated glial cells by neuronal damage can sensitize neurons by the release of pro-inflammatory cytokines and the chemokines such as Fractalkin (Tsuda et al., 2005; Shan et al., 2007). This interaction exemplifies the tight link between inflammation and nociception, beyond the well known and studied activity of inflammatory mediators on nociceptive nerve endings in clinically inflamed tissue. Neuropathic pain is frequently observed in patients with cancer, AIDS, lumbar
disc syndrome, herpes infection, traumatic spinal cord injury, multiple sclerosis, stroke and chronic diabetes patients.

3.2. DIABETES MELLITUS AND NEUROPATHIC PAIN

Diabetes Mellitus has been demonstrated to affect more than 100 million people worldwide in 2006 and is projected to affect more than 350 million by 2030 (Sarah et al., 2004). Both Type-1-Insulin Dependent Diabetes Mellitus (IDDM) and Type-2 Non-Insulin Dependent Diabetes Mellitus (NIDDM) are associated with the development of macro and micro-vascular complications such as retinopathy, nephropathy, cardiomyopathy and painful neuropathy (Ziegler et al., 2010). PDN is prevalent in chronic diabetic’s patients.

Early PDN is characterised by generation of unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia), and pain in response to a stimulus that does not normally provoke pain (allodynia), while loss of pain sensation, numbness, formation of gangrene are late symptoms of diabetic neuropathy (Ziegler et al., 2010). PDN is one of the most painful complications of diabetes mellitus, involving progressive neuronal damage and dysfunction, and up to 30% of patients with diabetes mellitus developed diabetic neuropathy (Davies et al., 2006). The neuropathic symptoms, including hyperalgesia, allodynia, hypoesthesia, and spontaneous pain, often develop in early stages, but may occur at any stage (Khara et al., 2007). These symptoms of diabetic neuropathy are highly unpleasant for the individuals and affect their quality of life (Hoffman et al., 2009; Connor, 2009) and constitute a considerable clinical problem and a burdensome condition worldwide (Davies et al., 2006; Hoffman et al., 2009). PDN is debilitating and often refractory to classical analgesics, including morphine (Raghavendra et al., 2004; Ibironke and Saba, 2006).
The mechanisms involved in genesis of diabetes-induced neuropathy are multifaceted and still remain poorly understood (Tesfaye, 2009; Ziegler, 2010). Numerous mechanisms have been suggested to be involved in painful diabetic peripheral neuropathy such as oxidative stress (Ozkul et al., 2010), formation of advanced glycation end-product (AGE) (Sugimoto et al., 2008), increased flux through the polyol pathway that leads to accumulation of sorbitol and fructose (Chen et al., 2010), myoinositol depletion and reduction in Na\(^+\)-K\(^+\)-ATPase activity (Pop-Busui et al., 2010), deficits in neurotrophism leading to reduced expression and depletion of neurotrophic factors such as nerve growth factor (Stuart et al., 2000), neurotrophin-3 and insulin-like growth factor (Shimoshige et al., 2010), as well as alterations in axonal transport (Kuwabara and Misawa, 2008) and PARP over-activation (Negi et al., 2010) (Fig-1). In addition to abnormalities of peripheral afferent nerves, altered sensory processing in the spinal cord may contribute to the development of diabetic neuropathic pain (Loseth et al., 2008; Morgado et al., 2010). Following nerve injury, neuropathic pain arises not only when these mechanisms are activated but also when peripheral and central mechanism sensitization is maintained (Tatsuro and Micho, 2006; Latremoliere and Woolf, 2009).

There is accumulating evidence that indicates direct effects of hyperglycemia on the spinal cord, which modify sensory processing and contribute to behavioral indices of neuropathic pain (Calcutt and Backonja, 2007; Morgado et al., 2010). Other factors, such as up-regulation of spinal excitatory glutamate receptors and increased release of glutamate and substance P, are also implicated in the development of diabetes induced spinal hypersensitivity (Anjaneyulu et al., 2008). Painful sensations are most commonly relayed via small primary afferents, the A-\(\delta\) fibres, and unmyelinated C fibres to the dorsal horn of the spinal cord; there is a synaptic junction in the outer part
of the dorsal horn and subsequently sensations are relayed to the spino parabrachial-amygdaloid pathway and spinothalamic tract.

The major consequences of hyperglycemia-induced nerve dysfunction are sensitization of nerve, sprouting of nerve fibre, central sensitization, tissue injury due to ischemic insult and inflammatory process (Suzuki et al., 2000; Selvarajah et al., 2006). Hyperglycemia induces morphological and structural changes in nerve, resulting in an increased excitability of damaged and surrounding neurons (Llewelyn et al., 1986). The structural changes in thinly mylinated A-delta fiber and unmyleinated C-fibre are well reported to be participating in the genesis of neuropathic pain. The neuronal hyperexcitibility arises in the primary afferent fibres and then spreads centrally. Accumulation of sodium (Na+) channels as well as the generation of new sodium channels that give rise to an excessive input into the central nervous system (Hong et al., 2004; Hong and Wiley, 2006; Brochu et al., 2006), causing central sensitisation in second-order neurons in the spinal cord and results in excessive neuronal discharge and signalling to CNS (Craner et al., 2002). Therefore, uncontrolled neuronal firing or hyper-excitability of nerve is attributed to increased expression of Na+ channels.

Following nerve injury, the voltage gated calcium (Ca++) channels are also increased (Gover et al., 2009; Su et al., 2010) and the increased Ca++ leads to increase in the release of substance P (SP) and glutamate (Zhang et al., 2009). SP and glutamate have a profound effect in the genesis of neuropathic pain (Anjaneyulu et al., 2008). The effectiveness of Ca++ channels blockers (pregablin and gabapentin) for treating PDN has been recently confirmed in various animal and clinical studies (Freeman et al., 2008). In addition, monoaminergic pathways, including the serotonergic, noradrenergic and to some extent dopaminergic systems originating in the raphe nuclei, locus coeruleus and dopaminergic nuclei in the mesencephalon, are implicated in PDN.
There is also some evidence to suggest that apoptosis of inhibitory neurons in the dorsal horn or spinal cord, which normally exert some inhibitory effect on second-order transmission neurons, results in spontaneous hyper-excitability in second-order neurons and in the descending inhibitory pathways.

It is, however, difficult to dissect the effects of diabetes on the peripheral and central nervous system. Evidence suggests that the impact of diabetes on the nervous system is far more generalised than previously thought (Conner et al., 2009). Various classes of drugs such as nonsteroidal anti-inflammatory drugs, antidepressants, anticonvulsants and opioids are currently under investigation in the management of diabetes-induced neuropathic pain but still there is no “gold standard” therapeutic approach or treatment to manage this difficult to treat pain (Tesfaye, 2009; Ziegler et al., 2010). The available treatments with these drugs are limited because of their partial effectiveness and analgesic resistance associated with severe potential toxicity (Ziegler et al., 2009). Therefore, a thorough understanding of molecular mechanism based therapeutic options and of the likely benefits and potential adverse effects of each option should be considered.

3.3. PATHOGENESIS OF PDN

3.3.1. Advanced Glycation End-products (AGEs) Pathway and PDN

Long standing hyperglycaemia has been reported to be involved in the formation of advanced glycation end-products (Lukic et al., 2008; Yamagishi, 2009). AGEs are heterogeneous modified intracellular and extracellular bio-molecules formed via a non-enzymatic reaction between reducing sugars and amine residues on proteins, lipids, or nucleic acids (Barbosa et al., 2008). Extracellular protein AGEs include plasma and matrix proteins that disrupt cellular adhesion and activate the receptor for AGEs (RAGE) (Yan et al., 2010).
Activation of RAGE or AGE–RAGE interaction induces oxidative stress (Wautier et al., 2001; Vincent et al., 2007; Yamagishi, 2009), PKC (Xu et al., 2010) and the transcription of nuclear factor kappa B (NF-κB) (Bierhaus et al., 1997; Toth et al., 2008). The promoter region of RAGE contains functional binding elements for NF-κB, and one consequence of NF-κB translocation is the up-regulation of RAGE itself (Li and Schmidt, 1997). NF-κB is a pleiotrophic gene regulator that regulates genes involved in promoting inflammatory reactions and neuronal dysfunction (Bierhaus et al., 2001; Haslbeck et al., 2005). Diabetic mice lacking RAGE showed significant improvement in PDN (Myint et al., 2006) and diminished expression of NF-κB and PKC as compared to wild type diabetic model (Lukic et al., 2008; Torreggiani et al., 2009). Collectively, the biochemical damage induced by AGEs results in increase ROS, impaired nerve blood flow and diminished neurotrophic support contributes to neuronal injury (Loseth et al., 2008; Yan et al., 2010).

3.3.2. Polyol Pathway and PDN

Increased flux, through the polyol-pathway leading to multiple biochemical abnormalities in the diabetic nerve, is thought to play a significant role in the pathogenesis of diabetic neuropathy (Sango et al., 2006; Takafumi et al., 2008). In polyol pathway, glucose is converted into sorbitol by aldose reductase (AR) and sorbitol dehydrogenase oxidises, sorbitol to fructose (Maria, 2005). Nicotinamide adenosine dihydrogen phosphate (NADPH) is consumed by aldose reductase-mediated reduction of glucose to sorbitol (Srinivasan et al., 2007) and NADPH is required for regeneration of antioxidant enzyme glutathione (GSH), thus deficient amount of glutathione contributes to oxidative stress (Kaneto et al., 2001). Moreover, conversion of glucose to sorbitol induces osmotic stress, and to restore osmotic equilibrium to cell, other osmolytes, particularly taurine and myo-inositol, are effluxes
from cells. Depletion of taurine and myo-inositol in nerve cells are implicated in PDN (Sima et al., 1997; Trevor et al., 2009) and supplementation of taurine and myo-inositol prevented neuropathic deficits (Pop-Busui et al., 2001; Trevor et al., 2009). On the other hand, excess formation of fructose in polyol pathway promotes advanced glycation end-product as well as depletes NADPH, further augmenting reactive oxygen species (ROS) mediated damage of cellular protein, lipid and neuron (Kaneto et al., 2001; Srinivasan et al., 2007; Sugimoto et al., 2008).

Aldose-reductase inhibitors (ARI), block the increased activity of aldose reductase, the rate-limiting enzyme that converts glucose to sorbitol (Ramirez and Borja, 2008), reduces sorbitol level implicated in PDN (Takafumi et al., 2008). Transgenic mice over-expressing aldose reductase in Schwann cells shown severe nerve conduction velocity deficit and oxidative stress under hyperglycaemic stress (Song et al., 2003). On the contrary, aldose reductase deficiency or inhibitors improves nerve conduction velocity deficits, Wallerian degeneration and nerve regeneration in diabetic animals (Chen et al., 2010). The first trials of ARIs in diabetic neuropathy were carried out 20 years ago and offer attractive therapeutic option to treat PDN (Ramirez et al., 2008). Later on, various compounds have been evaluated such as alrestatin, sorbinil, ponalrestat, tolrestat, epalrestat, zopolrestat and zenarestat for the treatment of PDN (Brown and Bird, 2004; Bril et al., 2009; Shimoshige et al., 2009). However, clinical trials with ARIs discomfited and shown lack of efficacy and potential toxicity. Epalrestat is the only ARI drug approved and marketed in Japan and India for PDN (Manish et al., 2007)

3.3.3. Hexosamine-Pathway and PDN:
The hexosamine pathway is activated when excess metabolite of glycolysis accumulated and was implicated in diabetes-induced oxidative stress and
complications (Hideaki et al., 2001). Fructose-6 phosphate is a metabolic intermediate of glycolysis. However, during glucose metabolism some fructose-6 phosphate is shunted from the glycolytic pathway to the hexosamine pathway and is converted to glucosamine-6 phosphate by glutamine fructose-6 phosphate amidotransferase (GFAT) (Srinivasan et al., 2007). The end-product of this pathway, UDP-N acetyl-glucosamine (UDP-GlcNAc), is a substrate for the glycation of important intracellular factors including transcription factor, thereby affecting the expression of many genes including plasminogen activator inhibitor (PA-1) and transforming growth factors (TGF) and leads to diabetic micro-vascular complications (Du et al., 2000; Hideaki et al., 2001). Inhibition of GFAT block the transcription of TGF and PA-I and are beneficial in PDN (Maria, 2006; Srinivasan et al., 2007). In addition, the hexosamine biosynthesis inhibitor azaserine prevents endothelial inflammation and dysfunction under hyperglycemic condition through antioxidant effects (Angana et al., 2009).

3.3.4. Protein Kinase C, Pathway and PDN

Hyperglycemia has been reported to enhance the expression of PKC (Iskandar, 2006). There are twelve isoforms of PKC, identified according to their structure and co-factor requirements. Activation of PKC is mediated primarily through increased release of diacylglycerol (DAG). The PKC pathway is an additional mechanism implicated in hyperglycemia induced painful neuropathy (Evcimen and King, 2007; Geraldes and King, 2010). Increased glucose levels stimulate diacylglycerol (DAG), which in turn activates PKC and PKC-β (Igwe and Chronwall, 2001; Geraldes and King, 2010). A highly selective and orally active PKC-β isoform-inhibitor, ruboxistaurin, has been developed and tested in PDN. Administration of ruboxistaurin ameliorated several neuropathic deficits in experimental diabetic neuropathy (Carolina et al., 2007; Danis and Sheetz, 2009; Xu et al., 2010). Moreover, Ruboxistaurin mesylate also attenuates
overexpression of transforming growth factorβ (TGF-β) (Richard et al., 2007), osteopontin expression, macrophage recruitment, and tubulointerstitial injury in advanced experimental diabetic nephropathy (Kelly et al., 2005).

**Fig-1: Possible mechanisms involved in the genesis of PDN.**

Hyperglycemia-induced generation of mitochondrial ROS, sorbitol and formation of AGEs initiates a vicious circle by activating stress-sensitive pathways such as NF-kB, p38 MAPK and Jak/STAT, polyol (sorbitol) and hexosamine pathways, PKC and RAGE. Glycation of protein, lipid nucleic acid by production of AGEs, sorbitol and pro-inflammatory cytokines exerts a positive feedback on ROS and RNS synthesis and potentiates PKC-mediated vascular dysfunction by altering gene expression as well as vascular function and structure.
Abbreviations: MAPK, mitogen activated protein kinase; PKC, protein kinase-c; NO, nitric oxide; AGE/RAGE, advanced glycation end product (receptor); ROS, reactive oxygen species; TGF, transforming growth factor; PAI, plasminogen activation inhibitor; NF-kB, Nuclear factor kappa-B

3.3.5. Growth factors and PDN

PDN is characterized by neuronal degeneration and damage to supporting Schwann cells, perturbations in growth factors such as nerve growth factor (NGF) (Hellweg et al., 1990a; Leinninger et al., 2004), insulin-like growth factor (IGF) (Ishii and Lupien, 1995), and neurotrophin 3 (NT-3) have been suggested to be involved in the pathogenesis of diabetic neuropathy.

NGF selectively promotes the survival, differentiation and maintenance of small fibre sensory and sympathetic neurons in the peripheral nervous system (Recio-Pinto and Ishii, 1988). The receptors for the NGF family of growth factors consist of the p75NTR and a specific trk tyrosine kinase, which confers ligand specificity. It has been reported that the endogenous level and expression of growth factors are altered in animal models of DPN (Hellweg et al., 1991b; Ishii and Lupien, 1995). Moreover, in multiple diabetic models; retrograde transport of NGF was diminished (Hellweg et al., 1994). Interestingly, when glucose levels return to normal, NGF levels also return to normal. This indicates that diabetes, either due to hyperglycaemia or by lack of insulin, has the capacity to regulate growth factors (Hellweg et al., 1991)

3.3.5.1. Insulin-like growth factors (IGFs)

IGFs are produced in the kidney, spinal cord, skeletal muscle and peripheral glia. IGFs are neurotrophic factors capable of supporting neurite outgrowth and survival in a wide variety of peripheral and central neurons (Ishii and Lupien, 1995; Recio-Pinto
Systemic IGF-I levels are reduced in rats with STZ-induced diabetes (Migdalis et al., 1995; Boni-Schnetzler et al., 1989) and are lower than those in diabetics without PDN. In this model of IDDM, IGF mRNA content is reduced in nerves (Wuarin et al., 1996), liver and spinal cord (Ishii and Lupien, 1995). Moreover, abnormal expression and levels of circulating IGFs and/or changes in expression of receptors for IGF were observed in diabetic human subjects (Sean et al., 2003), in STZ-rats (Armstrong et al., 2000), and in the type 2 diabetes Zucker diabetic fatty (ZDF) rat model (Kobayashi and Kamata, 2002). Furthermore, in obese Zucker rats, both insulin- and IGF-I resistances were shown to develop and mediate impaired glucose tolerance in these models of pre-diabetes. This suggestion is supported by observations of recovery of NCV and reversion of atrophy of myelinated sensory axons in the sural nerve of STZ rats treated with intrathecal IGF-I (Ekstrom et al., 1989; Zhuang et al., 1996). Replacement of IGF prevents neuropathy in diabetic nerves in the absence or even in the presence of persistent hyperglycemia (Ishii and Lupien et, 1995; Migdalis et al., 1995). Nerve regeneration is severely blunted in diabetic rats (Ekstrom et al., 1989) and restoration of insulin restores nerve regeneration as well as circulating IGF-I. Direct application of IGF-I to the site of nerve crush in diabetic rats resulted in restoration of sensory regeneration and a prevention of hyperalgesia (Zhuang et al., 1996).

### 3.3.5.2. C-Peptide

Similar to IGFs, reduced level of C-peptide has been implicated in the pathogenesis of PDN (Wahren et al., 2007). C-peptide is a segment of the pro-insulin molecule sliced off to form insulin and acts through both its own receptors and modulating activity of insulin receptors (Johansson et al., 1996 & 2000). It has been shown that C-peptide, in contrast to previous belief, possesses the characteristics of a bioactive peptide. C-
peptide binds specifically to various cell membranes, including endothelial, renal and nerve cells (Johansson et al., 1996), with subsequent activation of an intracellular signaling cascade resulting in stimulation of endothelial nitric oxide synthase (eNOS) and Na⁺,K⁺-ATPase (Johansson et al., 2001; Ekberg and Johansson, 2008). Moreover, recent data indicate that C-peptide stimulates several transcriptional factors, as well as several neurotrophic factors. Moreover, it has been demonstrated that exogenous administration of C-peptide in replacement dose to patients lacking endogenous C-peptide results in restoration of reduced blood flow in several tissues and improvement of renal and nerve function (Pierson et al., 2003). It has been reported that C-peptide also enhances auto-phosphorylation of IR and effects of insulin, and treatment with C-peptide reverses decreased expression of IGF-I, NGF and neurotrophin-3 receptors in type 1 spontaneously diabetic rats (Apfel et al., 1994). In neuropathy, it has been shown to have effects on Na+/K+-ATPase activity, expression of neurotrophical factors and improve diabetes-induced reduction in endoneural blood flow (Ekberg and Johansson, 2008).

3.3.5.3. Vascular Endothelial Growth Factor (VEGF): VEGF was originally discovered as an endothelial specific growth factor with a prominent role in angiogenesis and retinopathy (Aiello et al., 1994). However, recent observations suggest that VEGF has direct effect on neurons and glial cells stimulating their growth, survival and axonal outgrowth (Sondell et al., 1999). It has been shown that reduction in VEGF activity in STZ-diabetic mice results in the failure of neovascularization in hypoxic tissue in the lower limb (Rivard et al., 1999; Veves and King, 2001; Anghel et al., 2007).

STZ-diabetic rats are associated with marked destruction of the vasa nervorum of the sciatic nerve and that both the neuropathy and loss of vasa nervorum may be
successfully modified by VEGF gene transfer (Schratzberger et al., 2001). Moreover, after 4 weeks intramuscular gene transfer of plasmid encoding VEGF-1/2 nerve vascularity, blood flow and both large and small fibre dysfunction was restored in STZ treated diabetic rats. Recently, polymorphism of the VEGF gene at position −7C/T has been implicated in the pathogenesis of diabetic neuropathy as it may harbour some functional/regulatory potential in VEGF gene expression (Tavakkoly-Bazzaz et al., 2010).

3.3.5.4. Nerve Growth Factor (NGF): NGF is the most studied growth factor in PDN (Pittenger and Vinik, 2003). NGF is produced by muscle and keratinocytes, and its trkA receptor and was trophic to sensory and sympathetic neurons (Schmidt et al., 2000). NGF levels are reduced in diabetic nerve as well as retrograde transport of NGF is diminished (Lee et al., 1992; Hellweg et al., 1994; Fernyhough et al., 1994). However, when glucose levels return to normal, NGF levels also return to normal and attenuate the behavioural symptoms of PDN (Unger et al., 1998). Some studies have generated conflicting results with regard to NGF expression levels. Despite these discrepancies, an observed decrease in the retrograde transport of NGF (both endogenous and exogenous) in diabetic rats is noteworthy, in that the transport of NGF to the soma is required for its neurotrophic effects to occur (Hellweg et al., 1994; Anand et al., 1996; Vinik, 1999). However, a 6 month phase II trial with recombinant human NGF (rhNGF) in 250 patients with symptomatic diabetic neuropathy showed an improvement of the sensory component of the neurological examination and both cooling detection and heat as pain threshold, but no effect on neuropathic symptoms (Zochodne and Said, 1998; Freeman, 1999). In contrast, a subsequent large 12 month phase III trial failed to demonstrate a favourable effect of rhNGF on subjective or objective variables of diabetic neuropathy.
Another, trophic factor NT-3 is expressed in muscle and skin and has been shown to be necessary for the development of muscle spindle and Merkel cell afferent nerve fibres in animal models (Andreassen et al., 2009). It can signal through trkA and B to some extent, and primarily signals through trkC, suggesting broad therapeutic potential (Tomlinson et al., 1997). Like trkB, trkC is found in motor neurons and a population of large-diameter sensory neurons responsible for proprioception and tactile sensation (Yajima et al., 2002). NT-3 proteins levels are upregulated in diabetic sural nerve, though mRNA levels have been reported as both increased and decreased (Mizisin et al., 1999; Sayers et al., 2003).

3.3.6. Oxidative Stress and PDN:

Increased oxidative stress is a unifying mechanism in the causation of DM and diabetes-induced complications (Vincent et al., 2010). Antioxidant treatments have proven benefits in DM and its complications (Pazdro and Burgess, 2010). Long standing or persistent hyperglycaemia causes increased production of free radicals, especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation (Vlassara and Striker, 2010). Moreover, diabetes produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation, which is responsible for increased incidence of atherosclerosis and other cardiovascular disorder, that are major complication of diabetes mellitus (Johansen et al., 2005).

The increase in the level of ROS i.e superoxide (O2), hydrogen peroxide (H2O2) and hydroxyl radicals (‘OH/HOCL) in diabetes could be due to their increased production and/or decreased destruction by non-enzymic and enzymic catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD), antioxidants that contribute to eliminate ROS (Friederich et al., 2009). ‘O2 is immediately converted
into $H_2O_2$ by manganese superoxide dismutase (Mn-SOD) in the mitochondria and by copper (Cu)-SOD in the cytosol. $H_2O_2$ is then converted to $H_2O$ and $O_2$ by GSH-Px or CAT in the mitochondria and lysosomes, respectively (Vincent et al., 2010). The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes (Rosen et al., 2001; Robertson, 2004; Johenson et al., 2005). Also, this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defences (Robertson, 2004).

Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus, are thought to be the principle etiology of chronic diabetic complications, including PDN (Friederich et al., 2009; Pazdro Burgess, 2010). Free radicals are continually produced in the body as a result of normal metabolic processes and interaction with environmental stimuli. Implication of oxidative stress in the pathogenesis of diabetes and its complication is suggested, not only by oxygen free-radical generation, but also due to nonenzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, lipid peroxides formation and decreased anti-oxidant enzymes levels (Johansen et al., 2005; Friederich et al., 2009; Vincent et al., 2010).

In normal neuron, ROS production is tightly regulated. Under normal conditions, ROS is quickly eliminated by antioxidant defence mechanisms. Mitochondria in neuron is sensitive to oxidative damage which results impaired energy regulatory function that leads to loss of neuronal function and the development of PDN (Friederich et al., 2009; Vincent et al., 2010; Yamagishi, 2009). Moreover, excess generation of mitochondrial ROS due to hyperglycemia initiates a vicious circle by activating stress-sensitive pathways such as NF-κB, p38 MAPK, Jak/STAT, PKC and
pro-inflammatory cytokines that contribute to diabetic complications (George, 2008; Ziegler et al., 2009). In addition, hyperglycaemia increases the formation of potent oxidant peroxynitrite, which is formed by the combination of superoxide anion radical with nitric oxide, and the formed peroxynitrite has been documented to play a key role in experimental and clinical diabetic neuropathy (Obrosova et al., 2005a & 2005b; Obrosova et al., 2007; Drel et al., 2010).

3.3.7. Spleen/ Spleen Derived Factor (s) and PDN

Spleen is the principal organ of immune system contains more lymphocytes than all lymph nodes combined. It is functionally involved in immunity including phagocytosis and also in storage of erythrocyte. Splenic macrophages form a large part of mononuclear phagocytic system of the body. It removes microbes, tissue debris, and other particulate matter, aged erythrocyte and platelet circulating in blood. Moreover, spleen is involved in phagocytosis of circulating antigens and for the formation, initiation of humoral and cellular immune response.

Thymus gland and spleen are the principle organs of immune system and a rich source of cytokines. Neonatal thymectomy has been reported to prevent the development of NIDDM (Like et al., 1982: Ogawa et al., 1985; Jeroen et al., 2004). It has been demonstrated that spleen or spleen derived factor(s) modulate pain threshold (Kamei et al., 1992: Khan et al., 2009). Spleen derived factor(s) is also reported to be involved in diabetes induced morphine analgesic tolerance (Kamei et al., 1992 & 1995b: Taliyan et al., 2010a). Splenectomy or splenectomised beije-j mice regain the antinociceptive effect of analgesic and transfer of their mononuclear cells to their normal litter-mate’s decreased antinociceptive effect of morphine (Kamei et al., 1992: 1994a & 1994b: 1995a: 1996; Taliyan et al., 2010a & 2010b) and DAMGO, a µ opioid agonist (Kamei et al., 1994a). Moreover, the enhanced spontaneous locomotor
activity observed in diabetic mice is markedly reduced in mice that are splenectomised either before or three days after STZ injections. These observations strongly implicate that spleen or factor(s) derived from spleen mononuclear cells may be involved in decreasing the sensitivity to analgesic effect of opioids in diabetic animals.

3.3.8. Cytokines and PDN

Type I -insulin-dependent diabetes (IDDM) is an autoimmune disease characterised by specific and progressive loss of β-cells (Yoon and Jun, 1998 & 2003) with an unknown etiology but with a definite outcome, resulting in the progressive misdirected immunologic destruction of insulin-secreting pancreatic β - islet cells by autoreactive leukocytes and their mediators (Atkinson et al., 1994; Bach, 1994). Although, the precise mechanism of the genesis of the disease remains unclear; a combination of genetic, immunologic, and nongenetic factors contributes to the onset and progression of IDDM (Lipton et al., 1992). It has been reported that specific HLA antigens, in particular DR3 and DR4, have been associated with increased risk for IDDM development (Lipton et al.,1992: Tomer et al.,1997), while DR2 alleles generally have been described as “protective” of IDDM (Thorsby et al.,1993). In addition to HLA predisposing factors, viral infection, psychological factors (Riazi et al., 2004), and dietary factors (Howard, 2002) among others, have been described as predisposing factors. Accumulating line of evidence indicate that the frequent coexistence of IDDM with immune disorders, results from an inherent dysregulation in humoral immunity and cell-mediated immunity (Atkinson et al., 1994: Beyhum et al., 1997). This is further supported by the presence of autoreactive antibodies targeting selectively B-cell of Islet and other autoantigens (Figueroedo et al., 1996: Hagopian et al., 1993), circulating autoreactive T cells (Wang et al., 1987a), heightened expression of adhesion molecules (Itoh et al., 1993), reduced levels of serum cytokine inhibitors
(Balasa and Sarvetnick, 1996), and sustained expression of cytokines and their high-affinity receptors (David et al., 2000; Goldberg, 2009).

The involvement of T-cell- and macrophage-derived cytokines in IDDM pathogenesis were largely based on studies with the genetically IDDM-predisposed nonobese diabetic (NOD) mice and Bio-Breeding (BB) rats, animal models which display many of the characteristics of human type I diabetes, and have focused on direct cytotoxic and indirect immunomodulatory effects of cytokines in mediating β-destruction (Rabinovitch and Suarez-Pinzon, 1998; Rabinovitch, 2003; George, 2008). The excess release of insulin or hyperinsulinemia or cytokines from virus infected β-cells recruit macrophages to the islets (Fernandez-Real and Ricart, 1999). These macrophages and dendritic cells present in islets recognise β-cells-autoantigens. It activates T-helper (Th) cells, which may induce cytokines transcription and consequently add to the pool of cytokines in the islets (Rabinovitch and Suarez-Pinzon, 1998; David et al., 2000).

Numerous animal studies indicate that Th1 cytokines exacerbate (pro-inflammatory cytokines), while Th2 cytokines protect from IDDM (Lenschow et al., 1996; Marselli et al., 2001). However, contrary evidence is accumulating which demonstrates that the progression of IDDM from insulitis (pancreatic mononuclear cell infiltration) to frank hyperglycaemia is under the control of both Th1 and Th2 cells and their respective cytokines (Katz et al., 1995; Duncan and Swain, 1994). Th1 cytokines induce Th1 activity and block Th2 activity, whereas Th2 cytokines promote Th2 activity while inhibiting Th1 activity (Murata et al., 2002). This indicates that induction of one Th0 program is accompanied by a corresponding decline in the activation of the other Th program. In any event, difference in cytokine secretion between Th1 and Th2 cells translates into functional differences, as Th1 cells, by producing IFN-γ, activate CD81 T cells and macrophages and promote cell-mediated immunity (Marselli et al.,
On the other hand, Th2 cells stimulate IgM, IgG1, and IgE synthesis by B cells and activate eosinophils, thus promoting hypersensitivity reactions due to their capacity to produce IL-4 and IL-5 (Charles, 2005). In view of their role in macrophage activation and induction of delayed-type hypersensitivity reactions, Th1 cells are regarded as pro-inflammatory, while Th2 cells, which inhibit Th1 activity, were considered anti-inflammatory. Consistent with this characterization were the findings that IL-4 and IL-10—exclusive products of Th2 cells—inhibited IL-2-mediated responses and suppressed the production of the Th1 cytokines (TNF-α, IL-2, IFN-γ). The Th1 primes cytokines play a direct role in the pathogenesis and progression of IDDM, while Th2 cytokines should afford protection against Th1-mediated destruction of β- islet cells (Marselli et al., 2001). Therefore, IDDM was associated with an increase in the expression of Th1 cytokines and a corresponding decline in the production of Th2 (IL-4, IL-6 and IL-10) cytokines (Serreze et al., 2001b). Destruction of β-cells was suggested to be due to a frank Th1-driven insulitis, and it was suggested that IDDM could be abrogated by induction of Th2 cytokine expression or by treatment with the Th2 cytokines IL-4 and IL-10 (Marselli et al., 2001), which act through inhibition of the production of Th1 cytokines. Th1 cytokines, including IFN-γ, exerted their effects primarily at the level of macrophage and CD8+ T-cell activation, enhancing of these cells into the islets, thus accelerating b-cell destruction through the release of preformed and de novo-synthesized cytotoxic mediators (nitric oxide, oxygen radicals, serine esterase’s, etc.) (Murata et al., 2002; Koch et al., 2007).

However, recent reports argued against this oversimplification as Th2 cells and their mediators were shown to be involved in IDDM pathogenesis through facilitation of pancreatic mononuclear-cell infiltration and acceleration of β- islet cell destruction (Pakala et al., 1997; Sami et al., 1999).
Higher levels of pro-inflammatory cytokines correlate with the incidence of diabetic neuropathy (Doupis et al., 2009). Spinal pro-inflammatory cytokines such as TNF-α, IFNγ and ILs are powerful pain-enhancing signals that contribute to neuropathic pain (George, 2008; Doupis et al., 2009). A major feature of chronic inflammation and neuropathic pain is an increased synthesis of nitric oxide and prostaglandin and this is stimulated by a number of cytokines that are present at the injury or inflammatory sites including TNF-α, IFNγ and ILs (Maedler et al., 2002; Koch et al., 2007; Goldberg et al., 2009).

3.3.8.1. Biology of Tumour Necrosis Factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α) was originally discovered as a monokine produced by macrophages. It was subsequently revealed that various cells, such as fibroblasts, epithelial cells, adipocytes, and myocytes, also produce TNF-α, which has a variety of biological activities (Morohoshi et al., 1996). The release and synthesis of TNF-α is stimulated in monocyte or macrophages by many different exogenous substances such as lipopolysaccharide (LPS) and β-glucones or by endogenous mediators such as IL-1. TNF-α is synthesized as membrane bound pro-protein, comprising of 233 amino acids with a molecular weight of 26 kDa (Tang et al., 1996). The pro-protien is cleaved by a specific metalloprotease, also called as TNF-α-converting enzyme (TACE) (Black et al., 1997), to yield a 212-amino acid-long type II transmembrane protein, arranged in stable homotrimers.

3.3.8.1.1. Receptors for TNF-α

Two receptor are recognized for TNF-α; a high affinity cell surface receptor TNFR-1 (CD 120a, 55-60 kDa), which is expressed almost all cells and TNFR-2 (CD 120a, 75-80 kDa), which is expressed on haemopoietic and endothelial cells. The affinities of TNFR-1 and TNF-2 for soluble circulating TNF-α are similar. When TNF-α trimer
binds to TNFR-1, a number of actions are initiated such as cytotoxicity, fibroblast proliferation, synthesis of prostaglandins, and up regulation of adhesion molecules, nuclear factor kappa-b site in the enhancer element of the light chain immunoglobin k gene (NF-kB) and transcription factor activation (Gaur and Aggarwal, 2003). The function of TNFR-2 is not well understood but it has been suggested that it concentrate soluble TNF-α. Signaling through TNFR-I require intracellular domain of TNFR-2 suggesting that TNFR-2 activate the function of TNFR-I (Nishimura et al., 2003). Upon contact with their ligand, TNF receptors also form trimers, their tips fitting into the grooves formed between TNF monomers. This binding causes a conformational change to occur in the receptor, leading to the dissociation of the inhibitory protein SODD from the intracellular death domain. This dissociation enables the adaptor protein TNF alpha receptor activated dead domain (TRADD) to bind to the death domain, serving as a platform for subsequent protein binding (Zhao et al., 2003).

Following TRADD binding, various pathways can be initiated including NF-kB (Bouwmeester et al., 2004), MAPK (Li et al., 2005) and induction of death pathway (Zhao et al., 2003). NF-κB is a heterodimeric transcription factor that translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors (Cameron et al., 2008). Of the three major MAPK cascades, TNF induces a strong activation of the stress-related JNK group, evokes moderate response of the p38-MAPK, and is responsible for minimal activation of the classical ERKs (Li et al., 2005). Like all death-domain-containing members of the TNFR superfamily, TNF-R1 is involved in death signaling (Zho et al., 2003). However, TNF-induced cell death plays only a minor role compared to its overwhelming functions in the inflammatory process.

3.3.8.1.2. TNF-α and PDN
An increased level of TNF-α in diabetes is confirmed in preclinical and clinical studies. It is well reported that TNF-α plays a role in the pathogenesis of not only type 1 diabetes mellitus (Morohoshi et al., 1996; Coughlan et al., 2001) but also in type 2 diabetes mellitus (Moller et al., 2000). The mechanisms of enhanced TNF-α production may be ascribed to macrophage stimulation by high glucose itself (Morohoshi et al., 1996), hyperglycemia-induced oxidative stress (Guha et al., 2000), or exposure to advanced glycation-end products (AGEs) (Rashid et al., 2004). The increased production of TNF-α in vivo may exacerbate insulin resistance (Moller et al., 2000) and eventually promote diabetic complications, including PDN (Satoh et al., 2003; González-Clemente et al., 2005).

TNF-α is locally produced by SCs and has a role in peripheral nerve regeneration and regulation of apoptosis (González-Clemente et al., 2005). It has been reported that the levels of mRNA expression of TNF-α, as well as initiator and executive caspases are enhanced in early stages of diabetes (Guha et al., 2000). Activation of one of the major executive caspases, caspase-3, was also observed. Moreover, TNF-α has been detected at the injury site and shows temporal up-regulation in chronic constriction injury (CCI) of sciatic nerve in rats and raised levels of TNF immunoreactivity in dorsal root ganglia (DRG) of both injured and uninjured ipsilateral adjacent afferents, as well as of contra-lateral uninjured counterparts, which can only be partly explained by retrograde axonal transport (Zelenka et al., 2005). There is also a corresponding up-regulation of TNFR1 and TNFR2 in both nerve and DRG, with a temporal pattern of increased TNF mRNA expression, first in sciatic nerve, and then in DRG (Zelenka et al., 2005).

These studies suggest a role of TNF-α in regulation of apoptosis in diabetic neuropathy, inflammatory or immunological disease. This lead too much effort
recently in finding ways to down regulates its production or inhibits its effects (Marques et al., 1999). Inhibition of TNF-α synthesis can be achieved by inhibition of its gene transcription, decrease of mRNA and inhibition of translation (Whitehouse, 2004; Marques et al., 1999). It was demonstrated that cAMP–elevating agents suppress TNF-α synthesis in murine macrophages (Dorazil-Dudzik et al., 2004). Phosphodiesterase inhibitor agents such as rolipram, RO-201724, pentoxifylline and its analogue increase cAMP level and resulted in inhibition of TNF-α (Dorazil-Dudzik et al., 2004). Adenosine and its analogue, MDL 201112, have also been reported to inhibit TNF- production in activated mouse peritoneal macrophages (Parmely et al., 1993). Protein tyrosine kinase inhibitor including the pyrindinylimidazole have been shown to inhibit TNF-α and IL-1 in human monocyte (Geng et al, 1993).

A number of chimeric TNF-α antibody such as Adlimumab, Etanercept and CDP571 have been developed to treat conditions associated with elevated TNF-α (Scheinfeld, 2004). Thalidomide, a derivative of glutamic acid, inhibits TNF-α synthesis by decreasing the half life of TNF-α mRNA and was reported to possess various beneficial pharmacological properties including anti-inflammatory, immunomodulatory, and anti-angiogenic effects (Ribeiro et al., 2000; Ye et al., 2006). Thalidomide and thalidomide analogues have been reintroduced, despite its powerful teratogenic nature, as treatment for diverse chronic immunological/inflammatory diseases and it is suggested as a promising treatment for neurodegenerative diseases (Sampaio et al., 1991; Klausner et al., 1996; Ahuja et al., 2004). Thalidomide’s immunomodulatory effects are based on its capacity to modify T helper cell phenotype from a pro-inflammatory Th1 to an anti-inflammatory Th2 pattern, on the basis of the type of cytokines produced (Corrala and Kaplan, 1999). Recently, a large no of less toxic thalidomide analogue such as CC1069, CC1104 and CC1115 have been developed.
which are also effective inhibitors of TNF-α (Oliver et al., 1999; Frederick and Willium, 2004). Therefore, thalidomide or its analogue and pentoxiphylline may be used as effective TNF-α inhibitors.

### 3.3.8.2. Interleukins and Interferon – Gamma (IFN-γ)

Macrophages and dendrites are major sources of ILs, including IL-12 (Skeen et al., 1996). Daily administration of IL-12 has been reported to accelerate the onset of IDDM in young female NOD mice (Wegner et al., 2008). Enhanced IL-12 expression in pancreatic islets correlates with β-cells destruction (Yeshao et al., 2006). IL-12 has been shown to induce differentiation of Th-1 subset of CD4+ T-cells (Trembleau et al., 1997). Moreover, an increased expression of IL-12 in islets infiltrating mononuclear cells has been reported to correlate with the expression of Th 1 CD4+ and CD8+ T-cells derived pro-inflammatory cytokines such as TNF-α IL-1β, IFN-γ and IL-2 (Rabinovitch, 2003). Further, CD4+ and CD8+ T-cells-cells are reported to secrete IFN-γ and IL-2 (Henry et al., 2008). IFN-γ expression on pancreatic cell of NOD mice is reported to occur as a consequence of the mRNA expression of IL-18 and IL-12 (Simonian and Revzin, 2010). IFN-γ secreting splenic CD 4 + T-cells obtained from diabetic NOD mouse are noted increase the destruction of β-cells in recipient non-diabetic mice (Serreze et al., 2000). IFN-γ and IL-2 produced by CD4 + T-cells are known to activate CD 8 + T-cells and macrophages (Yoon and Jun, 1999). Transgenic NOD mice with over expression of CD8 + T-cells receptor have been reported to to exhibit an accelerated onset of IDDM (Nagata et al., 1994) and anti CD8+ immunoglobulin is demonstrated to prevent diabetes onset in mice (Chatenoud et al., 1997). Anti-IFN-γ polyclonal antibodies are reported to delay the onset of incidence of diabetes in BB rats (Debray-Sachs et al., 1991; Nicoletti et al., 1997) and transgenic mice (Gu et al., 1995).
Besides IFN-γ and IL-2, activated macrophages also secrete IL-1, which is reported to have inhibitory effect on mitochondrial energy production in islets. IL-1 is a pro-inflammatory and neuropoietic cytokine, locally produced by Schwann cells, endoneurial and infiltrating macrophages and fibroblasts in peripheral nerve, and sensory neurons in dorsal root ganglia (Skundric et al., 2002). IL-1β has been reported to inhibit insulin synthesis and accelerate onset of IDDM in mice (Welsh et al., 2005).

Pro-inflammatory cytokines such as TNF-α and IFN-γ augment β-cells cytotoxic effect of IL-1β. A polyclonal antibody and soluble IL-1 receptor significantly decrease the incidence of cyclophosphamide accelerated diabetes in male NOD mice (Nicolette et al., 1994; Gu et al., 1995; Cailleau et al., 1997).

These cytokines also activate NF-kB inducing kinases (NIK) which subsequently increase the expression of NF-kB (Cameron et al., 2008). NIK has been demonstrated to activate p38 and ERK sub family mitogen activated kinase (MAPK). Cytokines also activate ERK, JNK and p38 MAPK in β-cells of islets (Anderson, 2001). In addition, hyperglycaemia induced ROS and AGEs activate intracellular inflammatory signalling to up-regulate NF-κB (Toth et al., 2008). NF-kB is a pleiotrophic gene regulator that regulates the expression of various neuro-active diffusible factors including pro-inflammatory cytokines (Cameron et al., 2008).

Pro-inflammatory cytokines are known to increase the expression of iNOS (Koch et al., 2008). iNOS both induces and is induced by NF-κB, leading to a vicious cycle of inflammation (Iwasaki et al., 2007; Drel et al., 2010). The NO generated by iNOS directly modulates the blood supply to nerves and participates in macro and microvascular changes following injury (Obrosova et al., 2007; Vareniuk et al., 2008). NO has a direct role in axon and myelin breakdown following an injury (Pamela and Benjamin, 1998) and also contributes to the development of hyperalgesia and
allodynia (Grover et al., 2000; Chen et al., 2010). Genetic knockout nitric oxide synthase mice failed to display nerve-injury induced mechanical hypersensitivity (Guan et al., 2007). PDTC, a NF-kB inhibitor, prevents nitrite formation induced by simultaneous treatment with TNF-α IL-1β, IFN-γ and IL-2, suggesting a key role of NF-kB in the induction of iNOS by these cytokines (Malin et al., 1996; Giovanni et al., 2004). Moreover, cytokines induced NO plays a key role in priming β-cells for fas mediated destruction in IDDM (Stassi et al., 1997). Thus cytokines and NO seem to play a key role in the initiation and maintenance of neuropathic pain behavior symptom.

3.3.8.3. Cytokines inhibitor agents
Tacrolimus, mycophenolate and cyclosporine have been reported to prevent the synthesis and release of cytokines. Moreover, cyclosporine is known to modulate immune response and decrease NO level (Homayoun et al., 2002; Banafshe et al., 2005).

3.3.9. Nitric oxide (NO) and PDN
Accumulating evidence indicates that oxidative stress and nitric oxide (NO) is a key player in development of diabetes-induced painful neuropathy (Zheng et al., 2009; Friederich et al., 2009). NO is a free radical, synthesized from L-arginine, and an important vascular tone regulator. NO produces both physiological and pathological effects.

Physiological effects of NO take place when it is produced in minute quantities by constitutive nitric oxide synthases. NO mediates endothelium-dependent vasorelaxation by its action on guanylate cyclase in vascular smooth muscle cells (VSMC), initiating a cascade that leads to vasorelaxation and also displays
antiproliferative properties, inhibits platelet and leukocyte adhesion to vascular endothelium (Cardillo et al., 2000). Therefore, NO is considered a vasculoprotective molecule.

On the other hand, pathologic effects when reactive nitric oxide species (RNS) is produced in higher quantities and triggering a cascade of harmful events (Kozak et al., 2005; Friederich et al., 2009). RNS include free radicals like nitric oxide (\(\cdot\)NO) and nitrogen dioxide (\(\cdot\)NO2), as well as non-radicals such as peroxynitrite (\(\cdot\)ONOO), nitrous oxide (HNO2) and alkyl peroxynitrates (\(\cdot\)ONOO). Of these reactive molecules, \(\cdot\)O2-, \(\cdot\)NO and \(\cdot\)ONOO are the most widely studied species and play important roles in the diabetic complications, including painful diabetic neuropathy (Friederich et al., 2009; Drel et al., 2010). Furthermore, it has been shown that \(\cdot\)ONOO- oxidizes tetrahydrobiopterin (BH4), an important cofactor for NOS (Tiefenbacher et al., 2001; Alp and Channon, 2004), and causes uncoupling of NOS, which produces superoxide instead of NO (Verhaar et al., 2004; Bedard et al., 2007).

The enzymatic sources of augmented generation of reactive species in DM include NOS, NAD(P)H oxidase and xanthine oxidase (Ushio-Fukai, 2006). All isoform of NOS require five cofactors/prosthetic groups such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, BH4 and Ca2+-calmodulin. Deficiency or unavailability of NOS substrate such as L-arginine or one of its cofactors such as BH4, result in uncoupled state of NOS i.e production of superoxide instead of NO (Vasquez-Vivar et al., 1998; Verhaar et al., 2004). The switch from normal state to uncoupled is mainly determined through the availability of L-arginine and BH-4. NAD(P)H oxidase is another, membrane associated enzyme that consists of five subunits and is a major source of superoxide production (Bedard and Krause, 2007). The free radical superoxide is generated under hyperglycaemic condition,
rapidly combines with NO and formed a potent cytotoxic \( \cdot \text{ONOO}^- \) (Obrosova et al., 2004 & 2007; Friederich et al., 2009). Therefore, its chemical environment, i.e. presence of superoxide (\( \cdot \text{O}_2 \)), determines whether NO exerts protective or harmful effects.

\( \cdot \text{ONOO}^- \) causes nitration and nitrosylation, a condition called nitrosative stress, and reacts with a variety of biomolecules including proteins, lipids, and DNA, and has direct toxic effects on the nerve tissue leading to neuropathic pain (Kiss and Szabo, 2005; Obrosova et al., 2005a & 2005b & 2007). DNA single-strand breakage in response to oxidative/nitrosative stress has been implicated in poly (ADP-ribose) polymerase (PARP) activation (Negi et al., 2010).

### 3.3.10. Poly (ADP-ribose) polymerase pathway (PARP) and PDN

Oxido-nitrosative stress has been implicated in DNA single-strand breakage, followed by over activation of PARP, which contributes to PDN (Obrosova et al., 2005a; 2007d; Negi et al., 2010). PARP is a nuclear enzyme involved in a number of cellular processes, mainly DNA repair and programmed cell death. PARP is composed of four domains of interest: a DNA-binding domain, a caspase-cleaved domain, an auto-modification domain, and a catalytic domain. The DNA-binding domain is composed of two zinc finger motifs. In the presence of damaged DNA (base pair-excised), the DNA-binding domain will bind the DNA and induce a conformational shift. It has been shown that this binding occurs independent of the other domains. This is integral in a programmed cell death model based on caspase cleavage inhibition of PARP. The auto-modification domain is responsible for releasing the protein from the DNA after catalysis. PARP acts as a DNA-nick sensor and facilitates DNA repair by cleaving nicotinamide adenine dinucleotide (NAD\(^+\)) to nicotinamide and ADP ribose residues attached to nuclear proteins (Zdenko and Zhao-Qi, 2001; Olga et al., 2006). This result
in NAD\(^+\) depletion and the metabolic pathway that depends upon NAD\(^+\) such as glycolysis and mitochondrial respiration are impaired. Further, depletion of NAD\(^+\) leads to changes in gene transcription and expression, increased free radical and oxidant concentration, and diversion of glycolytic intermediates to other pathogenic pathways such as PKC, AGE formation and nitrosative stress (Mathews and Berk, 2008; Abraham and Rabi, 2009).

PARP also has the ability to directly induce apoptosis, via the production of PAR, which stimulates mitochondria to release apoptosis-inducing factor (AIF) (Yu et al., 2002). This mechanism appears to be calpain dependent (Vosler et al., 2009) and caspase-independent (Yu et al., 2002). PARP inhibition or gene deficient knock-out animals have been shown to counteract intra-epidermal nerve fiber loss and neuropathic pain in advanced diabetic neuropathy (Masutani et al., 2000; Sookja and Stephen, 2007; Obrosova et al., 2008). Moreover, concurrent targeting of nitrosative stress-PARP pathway has been reported to corrects functional, behavioral and biochemical deficits in experimental diabetic neuropathy (Negi et al., 2010).

### 3.4. PHARMACOLOGICAL TREATMENT FOR PDN

Various classes of drugs are being examined and used for the treatment of neuropathic pain and strict glycemic control remains the best preventive measure for neuropathy (Ziegler et al., 2009). The Diabetes Control and Complications Trial (DCCT) has reported that strict glycemic control in patients with DM not only decreased the incidence of neuropathy but also slowed its progression by 50-55% (DCCT, 1993; Pop-Busui et al., 2010). However, more than 30-40% patients are unable to achieve complete pain relief, even after glycemic control (Kaye et al., 2003; Ziegler et al., 2009). The patho-physiologic mechanisms that underlie these changes are not clearly understood, however, various proposed mechanisms are depicted in Fig-1. A thorough
understanding of molecular mechanism based therapeutic approach and of the likely benefits and potential adverse effects of each option should be considered. Newer agents have been designed to favorably influence the underlying process, rather than for symptomatic pain relief (Chong and Brandner, 2006; Cumbie and Hermayer, 2007). Approaches to prevention or treatment of diabetic neuropathy include the intensive treatment of hyperglycaemia, aldose reductase inhibition, anti-oxidant, cytokines inhibitors /antagonist and various symptomatic treatments (Ziegler et al., 2008a; & 2010b).

3.4.1. Anti-depressants and PDN

The First line therapy of drugs used to treat PDN are antidepressants (tricyclic antidepressants)(TCAs), anti-epileptics and selective serotonin reuptake inhibitors drug (SSRI) (Wong, 2008; Sibilia et al., 2009). TCA was studied for neuropathic pain in late 1950 and are known to act by inhibiting serotonin and nor-adrenaline reuptake. Several double blind, placebo controlled, crossover clinical trials have demonstrated the efficacy of the TCAs such as amitryptiline, imipramine, clomipramine, and desipramine (Max et al., 1991; Sindrup et al., 2003). Among them, amitriptyline /nortriptyline and desipramine were found to be effective and are considered first choice TCAs for treating painful diabetic neuropathy (Gilron et al., 2009). Although amitriptyline and desipramine relieve pain in many patients with painful diabetic neuropathy, side effects often preclude effective treatment (Max et al., 1991a).

The second generation antidepressant, SSRIs, fluoxetine, paroxetine, sertraline and citalopram, have not yet been FDA approved to treat painful neuropathy because they have been found to be no more efficacious than placebo in several controlled trials (Max et al., 1992 b). However, venlafaxine, a third generation TCA, has been demonstrated to produce significant pain relief as compared with placebo in a double
blind, placebo controlled study (Rowbotham et al., 2004; Kadiroglu et al., 2008). Duloxetine (Cymbalta), the first antidepressant drug, which equally inhibits reuptake of serotonin and nor-adrenaline, was approved by the FDA in 2004, for the treatment of diabetic neuropathic pain and fibromyalgia (Acuna, 2008; Sultan et al., 2008). However, TCA treatment is known to be associated with various side effects such as dry mouth, sweating, dizziness and sedation (Hall et al., 2010). In addition, recently, a retrospective study, including 58,956 person years follow-up on TCA therapy, indicates severe cardiac toxicity and TCAs are contraindicated in heart disease, epilepsy and glaucoma patients.

3.4.2. Anti-epileptics agents and PDN

Antiepileptic drugs originally developed for preventing seizure are now in broad use for the treatment of PDN (Sindrup et al., 2005). Carbamazepine was first anti-epileptic agents tested in late 1969 for neuopathic pain (Rull et al., 1969; Chakrabarti and Samantary, 1976). Carbamazepine and phenytoin are known to block the voltage gated sodium channels and both reduced PDN as compared to placebo (Jia et al., 2006), however, due to various side effect, their use for the treatment of PDN is not recommended (Yamada et al., 2002). Lamotrigine is another anticonvulsant reported to produce favourable results in the treatment of PDN (Vinik et al., 2007).

Lamotrigine has multiple actions; blockade of voltage gated sodium channels, decreased presynaptic calcium currents to inhibit the release of glutamate, and increased GABA levels in the brain (Vinik et al., 2007; Pop-Busui, 2007). Gabapentin and pregabalin, both produces analgesia via binding to the \( \alpha2-\delta \) site of L-type voltage gated calcium channels and decreasing calcium influx (Sibilia et al., 2009; Sandercock et al., 2009). Various multicentre double blind, randomized, placebo controlled trial, have demonstrated that extended gabapentin or gabapentin at a dose from 900 mg/day
to 3600 mg/day, significantly reduced pain of PDN compared with placebo (Sandercock et al., 2009; Chou et al., 2009).

3.4.3. Non-streoidal anti-inflammatory drugs (NSAIDs) and PDN

Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain through prostaglandin inhibition (Tegeder et al., 2001). The main COX inhibitors are the non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen, which can reduce pain and inflammation by blocking cyclooxygenase (COX) enzymes.

Different tissues express varying levels of COX-1 and COX-2. Although both enzymes act basically in the same fashion, selective inhibition can make a difference in terms of side-effects (Segev and Katz, 2004). The most frequent adverse effects of COX-inhibitors are irritation of the gastric mucosa and ulcer, a direct effect of inhibition of prostaglandin synthesis which normally has a protective role in the gastrointestinal tract (Raskin, 1999). Prostanoid synthesis is essential for the generation of inflammatory pain and this depends not only on prostanoid production at the site of inflammation, but also on the actions of prostanoids synthesized within the peripheral nervous system and central nervous system (CNS) (Graham and Hickey, 2003; Zhu and Eisenach, 2003). Novel pharmacological compounds that act along the pathway of prostanoid synthesis and action, both in the periphery and in the CNS, might provide increased benefit for treating inflammatory pain hypersensitivity (Hyperalgesia) (Reinold et al., 2005). COX-2 expression is elevated in the peripheral nerves in experimental diabetes and selective COX-2 inhibition in rats is protective against diabetes induced peripheral nerve dysfunction (Pop-Busui et al., 2002). Moreover, the transcription factor, nuclear factor-kappa B (NF-kB), regulates the expressions of
COX-2 (Lee et al., 2004). NF-κB has been reported to induce iNOS (Flodstrom et al., 1996; Pahan et al., 2000) and like COX-2, iNOS both induces and is induced by NF-κB, leading to a vicious cycle of inflammation (Haddad, 2007). NF-κB decoy or NF-Kb inhibitor drug such as pyrrolidine dithiocarbamate, significantly inhibited mechanical allodynia and thermal hyperalgesia following unilateral hindpaw inflammation evoked by complete Freund's adjuvant (CFA) (Gaku et al., 2001). These NF-κB inhibitors also suppressed the activation of spinal NF-κB and the subsequent markedly increase level of spinal COX-2 mRNA involved in neuropathy (Jobin et al., 1998; Narita et al., 2008).

Recently, NSAIDs have shown beneficial effect for the treatment of painful diabetic neuropathy (PDN) (Kellogg et al., 2005a; Kellogg et al., 2008c). Cox-2 selective inhibition and/or COX-2 gene inactivation provide protection against various neuropathy symptoms and deficits associated with PDN (Kellogg et al., 2007b). Elevated levels of nitric oxide (NO) produced by expression of iNOS and high levels of prostaglandins (PGs) generated by expression of inducible Cox-2 are important mediators of immune and inflammatory responses (Kellogg et al., 2008c). Moreover, a close interaction between NOS and COX pathways has become evident in inflammation and the cross-talk between these two pathways might be important in regulation of pain (Tetsuka et al., 1994). Therefore, inhibition or blockade of nitric oxide formation down-regulate COX and thereby decrease PGs production and provide pain relief in PDN (Kim et al., 2005; Dudhgaonkar et al., 2008). However, development of analgesic resistance and adverse effect associated with NSAIDs limits the use of these agents in diabetic neuropathy.

3.4.4. Opioids and PDN:
Opioids such as morphine, fentanyl, oxycodone are strong analgesics with proven efficacy to manage chronic pain (Pergolizzi et al., 2008; Riley et al., 2008) and WHO has recommended the use of opioids for treatment of chronic pain (Andrew, 2005). However, their use in treatment of PDN is still a matter of debate, some studies indicating usefulness of opioids in PDN (Shah and Carrig, 2004; Hays et al., 2005; Attal et al., 2010), whereas some other studies indicating inadequate efficacy of opioids in PDN (Karci et al., 2004; Christoph et al., 2010). Three opioids receptor, µ-opioid receptors (MORs), δ-opioid receptors (DORs), and κ-opioid receptors (KORs) have been isolated and cloned (Martin, 1983; Jordan et al., 2000). Several lines of evidence indicate the existence of physical and functional interactions between the opioid receptors, particularly between the µ and δ receptors (Traynor and Elliot, 1993). The physiological and pharmacological significance of µ-δ interactions have been substantiated by recent studies using opioid receptor gene knockout animals (Traynor and Elliott, 1993).

The existence of intermodulatory effects between µ and δ receptors has spawned a new interest in the pursuit of ligands with a mixed interaction profile at the µ and δ receptors as a novel therapeutic approach for the treatment of pain (Horan et al., 1992; Gomes et al., 2000). Chronic treatment with opioids are associated with side effect including constipation, urinary retention, impaired cognitive function, impaired immune function, and many other issues such as analgesic tolerance and addiction (Raghavendra et al., 2002).

It is well established that opioids are strong and effective antinociceptive drugs; however, their antinociceptive activity is decreased in diabetes associated neuropathic pain (Boulton, 2005). Moreover, hyperglycemia is demonstrated to be associated with desensitization/decreased functional expression of opioid (µ) receptor, which may
contribute to the genesis of diabetic neuropathy (Chen et al., 2002). Further, it has been reported that the antinociceptive effects of the i.c.v. administration of μ-opioid receptor agonists, such as morphine, [D-Ala2, N-MePhe4, Gly-ol5] enkephalin (DAMGO) and endomorphin-2, were reduced in diabetic animals (Kamei et al., 1992 & 1994b; Ohsawa and Kamei, 1997; Tasatargil and Sadan, 2004). On the other hand, the antinociceptive effects of i.c.v. administration of δ-opioid receptor agonists, such as [D-Pen2, D-Pen5] enkephalin (DPDPE) and (±) TAN-67 in diabetic mice were markedly greater than those in non-diabetic mice (Kamei et al., 1995; Chen and Pan, 2003).

Also, the antinociceptive potency of κ-opioid receptor agonist, U-50,488H, was not significantly reduced in diabetic mice as compared to non-diabetic mice (Kamei et al., 1992a), or was even enhanced in diabetic mice relative to non-diabetic mice (Suzuki et al., 2000).

These studies indicate that diabetic animals are hypo-responsive to the antinociceptive effect mediated by μ-opioid receptor, but were sufficiently sensitive to the antinociceptive effects mediated by δ- and/or κ-opioid receptors. By contrast, long acting opioids such as fentanyl, methadone and oxycodone were more effective than morphine in PDN (Hays et al., 2005; Riley et al., 2008). Recently, various randomized, placebo controlled study demonstrated that slow release oxycodone 80mg/day significantly relieved PDN (Gimbel et al., 2000; Hanna et al., 2008) and improved health related quality of life (Watson et al., 2003). Moreover, numerous clinical studies reported that pregablin and morphine/oxycodone prescribed together is more efficacious than one or either drug alone for alleviating PDN (Gilron et al., 2005; Zin et al., 2010). However, addiction and development of analgesic tolerance to opioids in nerve injury or diabetes-induced pain is the major hurdle in clinical practice.
(Raghavendra et al., 2004; Carolina et al., 2007). Repeated opioids administration causes spinal changes involving translocation and activation of protein kinase C and production of nitric oxide (NO) which may be involved in opioids–induced hyperalgesia and analgesic tolerance.

3.4.5. Cannabinoids and PDN:

The anecdotal use of Cannabis for therapeutically aim dates back to about 4000 years and cannabis is one of the oldest psychotropic drugs known to humanity. The most relevant are Cannabis sativa, Cannabis indica and Cannabis ruderalis. Cannabis sativa, the largest variety, grows in both tropical and temperate climates. The 1971 identification of the most active and clinically relevant component, \(\Delta^{9}\)-tetrahydrocannabinol (\(\Delta^{9}\)-THC) in C. sativa extracts, by Gaoni and Mechoulam, initiated a novel field of pharmacological study, most recently developing into investigation of the therapeutic potential of cannabinoids and related compounds.

Cannabinoids are the major active constituents of Cannabis sativa and are oxygen-containing aromatic hydrocarbon compounds (Pertwee, 1999). There are three major cannabinoids are Cannabidiol (CBD), Cannabinol (CBN) and Tetrahydrocannabinol (THC). There are two G protein coupled cannabinoid receptors have been identified by molecular cloning and named CB1 and CB2, so far (Matsuda et al., 1990). Shortly after the discovery of cannabinoid receptors CB1 and CB2, endogenous ligands including N-arachidonylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) were identified (Marzo, 1998). Activation of both receptor –CB-1 and CB-2 have been demonstrated to plays an important role in several pathways, including pain transmission (Walker and Hohmann, 2005), feeding (Marzo and Matias, 2005), and the rewarding effects of drugs like alcohol, tobacco, and cannabis (Thanos et al., 2005). CB1Rs couple primarly to G_{i/o} proteins, which in turn inhibit cAMP accumulation and
selective types of calcium channels, and activate mitogen-activated protein (MAP) kinase and a subset of potassium channels (Millns et al., 2001). CB1Rs are also activated by endocannabinoids such as anandamide and by exogenously administered compounds like the synthetic agonist-WIN55,212–2[(R)-(+)\-[2,3-dihydro-5-methyl-3-(4 morpholinylmethyl) pyrrolo [1, 2, 3-de]-1,4-benzoxazin-6-yl]-1 naphthalenylmethanone] or the naturally occurring delta(9)-THC [delta(9)-tetrahydrocannabinol], the psychoactive ingredient of cannabis. In addition, compounds such as CP55940 and HU210 are high efficacy agonists, whereas δ-9-tetrahydrocannabinol (δ 9-THC) is a partial agonist. Antagonists that are selective for the CB1 receptor include rimonabant (also known as SR141716), taranabant, AM251, AM281 and LY320135 (Pertwee, 2005; Howlett et al., 2009). A wide range of behavioural studies confirmed that cannabinoids are antinociceptive in animal models of chronic pain (Fox et al., 2001). The naturally occurring D9-tetrahydrocannabinol (D9-THC), cannabinol and synthetic cannabinoids agonist WIN55,212-2 and CP-55,940 inhibit responses to noxious thermal and mechanical stimuli in the hot-plate, tail-flick and paw pressure tests (Lichtman and Martin, 1991; Scott et al., 2004), as well as nociceptive behaviours in the formalin test (Tsou et al., 1996). Moreover, the endogenous CB1 receptor ligand anandamide is similarly antinociceptive in these tests (Fride and Mechoulam, 1993; Smith et al., 1994).

More recently, it has been shown that WIN 55,212-2 and anandamide are also effective against more persistent nociceptive processes, reducing thermal and mechanical hyperalgesia and mechanical allodynia following carrageen (Gutierrez, et al., 2007), capsaicin injection (Johanek et al., 2001; Patwardhan et al., 2006) or peripheral nerve injury (Guindon et al., 2007). In addition, the D9-THC analogue HU-
2111 has been shown to reduce autotomy induced by peripheral nerve section (Zalish and Lavie, 2003).

Both the analgesic and antihyperalgesic effects of cannabinoids are mediated by activation of CB1 receptors and are blocked by the selective antagonist rimonabant (SR141716A) (Marzo, 2009; Akopian et al., 2009). Likewise to CB1, activation of CB2 has been demonstrated to attenuate neuropathic pain in rodents (Racz et al., 2008). The identification of CB2 receptors in neuron and glial cells (Zhang et al., 2003; Beltramo et al., 2006) has opened new therapeutic approaches for CB2-ligands (Guindon and Hohmann, 2008). Selective, CB2 receptor activation induces analgesic effects in several animal models (Ibrahim et al., 2006; Whiteside et al., 2007; Giblin et al., 2007) and circumvents psychoactive side effects of CB1 agonists (Whiteside et al., 2007). Interestingly, CB2 receptor stimulation has been reported to attenuate neuropathic pain via suppression of microglial activation induced release of pro-inflammatory cytokines (Puffenbarger et al., 2000; Ehrhart et al., 2005; Romero-Sandoval et al., 2008). CB2 knock-out mice and wild-type littermates were exposed to sciatic nerve injury, and both genotypes developed a similar hyperalgesia and allodynia in the ipsilateral paw (Racz et al., 2008). Although some studies have suggested a potential role for CB2 receptors in the modulation of neuropathic pain, the mechanism underlying these analgesic effects and the exact involvement of CB2 receptors in the development of neuropathic pain has not been clarified yet. However, drug addiction and development of analgesic resistance and potential psychoactive adverse effect associated with cannabinoids, limits the use of these agents in neuropathy.

3.4.6. Neuro-sterioids and PDN:
The spinal cord is a biosynthetic center for neurosteroids, including pregnenolone (PREG), progesterone (PROG), and 3alpha/5alpha-tetrahydroprogesterone (3alpha/5alpha-THP) (Patte-Mensah et al., 2006). In particular, an active form of cytochrome P450 sidechain cleavage (P450scC) has been localized in sensory networks of the rat spinal cord dorsal horn. P450scC is the key enzyme catalyzing the conversion of cholesterol (CHOL) into PREG, the rate-limiting step in the biosynthesis of all classes of steroids.

Neuroactive-steroids, including progesterone, allopregnanolone, pregnenolone and dehydroepiandrosterone, represent steroid hormones synthesized de novo in the brain, that act as potent endogenous neuromodulators with rapid actions in the central and peripheral nervous systems (Gambhir et al., 2002; Christine and Ayikoe, 2008). In addition, neurosteroids are also synthesized either by glial cells, by neurons, or within the context of neuron-glia cross-talk (Jung-Testas et al., 1992 a; & 1999b; Ren et al., 2008). Various studies have suggested neurosteroids involvement in the control of neuron-degeneration and have reported to provide adequate neuroprotection that directly depends on their own capacity to produce neuroprotective neurosteroids (Baulieu, 1997; Leonelli et al., 2007).

Neurosteroids modulate several neurotransmitter systems such as γ-aminobutyric acid type A (GABA\textsubscript{A}), N-methyl-D-aspartate (NMDA) and acetylcholine receptors and attenuate inflammation induced pain and hyperalgesia (Ren et al., 2000; Gambhir et al., 2002). As physiologic consequences, neurosteroid are considered as crucial endogenous modulators of numerous physiological functions, including memory, neurogenesis and aging, neuronal plasticity, learning and memory processes, aggression, epilepsy and pain (De Nicola et al., 2009). Recently, neurosteroids has
been reported to have pain relief in experimental neuropathic pain model (Roglio et al., 2007; Leonelli et al., 2007) and also found to modulate pain threshold (Seo-Yeon et al., 2009). Neuro-active steroid treatments prevent peripheral myelin alteration induced by diabetes (Veiga et al., 2006). Moreover, neurosteroids such as testosterone and progesterone has been shown as neuroprotective in PDN (Leonelli et al., 2007; Roglio et al., 2007; Panahi and Sameni, 2009) and also enhanced antinociceptive effect of morphine (Winter et al., 2003). Antinociceptive effect of various analgesics has been reported to be sex dependent (Yu-Ching et al., 2009). Thermal hyperalgesia and allodynia development is profound in female as compared with male rats (Stoffel et al., 2005). Moreover, it has also been recently reported that the levels of neuroactive steroids present in the PNS and CNS of male and female rats are strongly affected by hyperglycemia (Pesaresi et al., 2010a & 2010b). It is interesting to highlight that incidence, progression and severity of diabetic complication, including neuropathy, show gender differences (Cheryl et al., 1992; Stoffel et al., 2005; Pesaresi et al., 2010a & 2010b). Further, the levels of neuroactive steroids such as, pregnenolone (PREG), progesterone (PROG) and its metabolites, testosterone and its metabolites, dehydroepiandrosterone (DHEA) are altered in cerebral areas (i.e., cerebral cortex, cerebellum and spinal cord) and in sciatic nerve of diabetic male and female rats (Melcangi et al., 2009; Pesaresi et al., 2011). Hormonal milieu, modulates mu, kappa, and delta opioid and cannabinoid receptor induced antinociception, in both male and female rats (Cheryl et al., 1992; Stoffel et al., 2005; Anaraki et al., 2008). Modulation of neurosteroid level in CNS as well in PNS as a consequence of persistent hyperglycemia or hypo-insulinemia may be involved in differentials effect of opioids, NSAID and cannabinoid in diabetic male and female. Moreover, both in PNS and CNS, the levels of Testosterone and its derivatives (i.e., dihydrotestosterone and
3alpha-androstanediol) are decreased by diabetes but these effects occur only in male. These observations strongly suggest that hormonal milieu affects pain perception (Stoffel et al., 2005). Effect of hyperglycemia induced modulation of neuro-steroid synthesis and levels in diabetic patients are still not well studied. Moreover, the effect of neurosteroid on pain sensitivity has not been explored so far.

**3.5 DIABETES-INDUCED NEURONAL HYPERSENSITIVITY AND ANALGESIC TOLERANCE**

Analgesics tolerance is a phenomenon whereby chronic or repeated exposure to a drug diminishes its antinociceptive effect, which creates the need for a higher dose to maintain the therapeutic effect.

At the molecular level at least three principal mechanisms can contribute to the development of analgesics tolerance to 7TM receptor ligands. These includes homologous receptor desensitization, 2) receptor down-regulation, and 3) heterologous desensitization. Homologous receptor desensitization is characterized by an uncoupling of the receptor from its G-proteins (Borgland et al., 2003; Virak and Williams, 2008). Receptor down-regulation involves a loss of receptor number caused by either receptor degradation or decreases in receptor synthesis, which results in fewer available ligand binding sites (Kohout and Lefkowitz, 2003; Shankaran et al., 2007). Heterologous desensitization is a disruption of signaling pathways in the cell that decreases the effectiveness of the agonist-occupied receptor to regulate the level of second messengers, even though receptor/G-protein coupling and receptor number may remain unchanged (Chuang et al., 1996: Kovoor et al., 1998). Any or all of these mechanisms can contribute to tolerance to a specific drug-receptor pair.
After activation, most 7TM receptors are desensitized and endocytosed and consequently, the receptors are either recycled back to the membrane—thus ready for a new encounter with a ligand—or degraded in lysosomes (Hamm, 1998; Cao et al., 1998; Gaidarov et al., 1999; Ferguson et al., 2001). As a consequence, the postendocytic fate of a receptor after exposure to a certain drug can determine what role internalization plays in modulating receptor availability and thereby signals transduction. Thus, elucidating the trafficking properties of a receptor can, by extension, provide insight into the mechanism(s) responsible for drug tolerance. For receptors that are recycled, endocytosis would not contribute to receptor down-regulation and would, in fact, promote resensitization of receptor function, possibly preventing tolerance (He et al., 2009). On the other hand, for receptors that are degraded, endocytosis would serve as the first step toward receptor down-regulation and perhaps promote tolerance (Kovoor et al., 1998; Martini and Whistler, 2007).

Accumulating evidence indicates development of antinociceptive tolerance after few days of administration of analgesics agent such as morphine (Ibironke and Saba, 2006), WIN 55,212-2, THC (Fan et al., 1994; Tappe-Theodor et al., 2007), dipyrrone and acetyl salicylic acid (Tsiklauri and Tsagareli, 2006) in both preclinical and clinical studies. As a result, the dose-response curve in analgesic-tolerant animals shifts toward the right relative to that for opioids, cannabinoids and NSAIDS-naive animals. However, mechanisms that underlie the establishment and maintenance of persistent pain, including diabetic neuropathy appear to overlap with those proposed for analgesics tolerance, including change in NMDA receptor activity, PKC activation and overproduction of cytokines and NO. The initiating event(s) in the development of analgesic tolerance to various analgesics remain to be elucidated. Accumulating evidence indicates that patients with nerve injury or PDN require higher doses of
analgesics because nerve-injury or diabetes-evoked hyperalgesia and allodynia mimic analgesic tolerance (Ragavendra et al., 2002a & 2004).

### 3.5.1 Opioids-Analgesic Tolerance in PDN:

Numerous studies have shown that the analgesic effect of opioids and its analogues was significantly decreased in diabetic laboratory animal as well as in diabetic patients (Ibironke and Saba, 2006). The mechanism involved in the development of analgesic resistance of opioids is complex and is yet not understood.

Numerous studies indicate that activation of either $\delta_1$ or $\delta_2$ opioid receptor subtype is required for the development of both antinociceptive tolerance to morphine and supersensitivity (i.e., enhanced morphine potency following chronic antagonist exposure) (Kamei et al., 1994). Blockadage of selective delta opioid receptors prevents the development of morphine tolerance and dependence in mice (Abdelhamid et al., 1991). Furthermore, decreased analgesic effect of morphine is attenuated in mice and rats by naltrindole and naltrindole 5′-isothiocyanate (5′-NT11) treatment, both selective antagonists at the $\delta_2$ opioid receptor. In addition, i.c.v. administration of antisense oligonucleotides that inhibit expression of the cloned $\delta$-opioid receptor (DOR) and deletion for the gene for DOR-1 (i.e., knockout mice) prevents development of analgesics tolerance. By contrast, synergistic effect of delta opioids receptor agonist with morphine has been demonstrated in laboratory animals (Rossi, et al., 1994; Gendron et al., 2007).

Likewise, much evidence implicates spinal dynorphin in the development of enhanced nociceptive sensitivity (hyperalgesia) that exaggerates antinociceptive tolerance to opioids (Vanderah et al., 2000). Although dynorphin A was originally identified as an endogenous antinociceptive and analgesic molecule (a $\kappa$-opioid agonist) (Nakazawa et
al., 1985), more recent studies indicate that dynorphin has significant pronociceptive activity that is not mediated by opioid receptors (Lai et al., 2001; Wang et al., 2001). Spinal administration of dynorphin antiserum (but not control serum) blocks the expression of abnormal pain behavior and the development of antinociceptive tolerance in rodents dosed chronically with opioids (Nichols et al., 1997). Precisely, how increased spinal dynorphin expression promotes pain and opioids tolerance is unclear. Whatever mechanism is involved, therapeutic strategies to diminish dynorphin release in the spinal cord could enhance opioids analgesic sensitivity in pathological pain states and reduce the development of antinociceptive tolerance (Vanderah et al., 2000; Gardell et al., 2002).

3.5.1.1 Anti-opioid Peptides: The antinociceptive effects of morphine are opposed by a number of endogenous peptides, termed anti-opioids, which are released in response to the administration of opioids (Wiesenfeld-Hallin et al., 1999). More than half of each dose of morphine given systemically to rats or humans is metabolized to an analgesically inactive metabolite, morphine-3-glucuronide (M3G) and to a lesser extent into morphine-6-glucuronide (M6G) (Smith et al., 1990). M3G is a potent neuroexcitant when administered by the i.c.v. route to rodents, evoking a range of excitatory behaviors including myoclonus, allodynia, wild-running, and seizures (Smith, 2000; Komatsu et al., 2009). Moreover, I.c.v. administration of M3G attenuates the antinociceptive effects of morphine consistent with the view that M3G is an anti-analgesic molecule (Smith et al., 1995; Vaughan and Connor, 2003). Indirect evidence in rats implicates M3G in the development of antinociceptive tolerance to systemically administered morphine via activation of NMDA receptors which is a common pathway involved in the development of morphine tolerance (Smith et al., 1990; Hemstapat et al., 2003). Thus, it is plausible that clinical analgesic
tolerance to morphine may, at least in part, result from an increasing accumulation of M3G in plasma and cerebrospinal fluid relative to morphine and M6G. In addition, some neuropeptides have been shown to have anti-opioid activity in behavioral studies in rodents e.g. vasopressin (Ratka and Kloet, 1988), oxytocin (Laorden et al., 1997), nociceptin (Sun et al., 2001) and neuropeptide FF (NPFF) (Lake et al., 1991; Gelot et al., 1998). The precise contributions of each of these anti-opioids to analgesic tolerance remain to be clarified.

3.5.2 Cannabinoids- Analgesic Tolerance in PDN: Numerous reports have demonstrated the development of tolerance after repeated or chronic treatment with cannabinoids agonist, including WIN55,212–2, delta(9)-THC and CP55,940 (Pertwee et al., 1993; Martini et al., 2004; Maguma et al., 2008). Although the specific mechanism mediating tolerance to cannabinoids remains unresolved, several studies have found CB1 receptor down-regulation after chronic administration (Wu et al., 2008).

Zhang et al (2007) demonstrated that high glucose concentrations are associated with decreased expression of CB1 receptors in nerve cells which may contribute to the neurodegenerative process observed in diabetes. In diabetic patients small fibre (C and A-δ) neuropathy is responsible for the early hyperalgesia and allodynia, and the late hypoalgesia, impairment of warm thermal perception and skin blood flow.

Activation of CB1 receptors suppresses neuropeptide release via inhibition of Ca2+ channels and activation of K+ conductance (Eva et al., 2000). Thus, anandamide can either inhibit or stimulate sensory neurotransmission, via the CB1 or TRPV1 receptor, respectively. There is some evidence to suggest that the balance of CB1- versus TRPV1-mediated responses is tipped unfavourably towards TRPV1 in diabetes (Pabbidi et al., 2008). Kamei and colleagues (2001) have reported that the thermal
hyperalgesia and allodynia observed in diabetic mice is due to sensitization of TRPV1 receptors.

Inhibition of vanilliod receptor prevents thermal hyperalgesia and allodynia (Kamei et al., 2001). Another key determinant of cannabinoid analgesics tolerance is G-protein-coupled receptor-associated sorting protein (GASP). It was demonstrated that GASP1 plays a major role in the postendocytic sorting of opioid and cannabinoid receptors to lysosomes, the degradative pathway, and contribute in the development of analgesics tolerance (Koch et al., 2005; Martini and Whistler, 2007; Martini et al., 2007). While receptor desensitization appears to be specific to selected brain areas, receptor down-regulation is a prominent cellular hallmark in tolerant animals and has been observed in multiple brain regions.

Sustained exposure of opioids significantly increased dynorphin level in spinal cord and promote pain sensitivity to thermal stimuli and opioids tolerance (Gardell et al., 2002). Similar to opioids, recent studies indicate that sustained cannabinoids administration produces increased expression of spinal dynorphin, which promotes enhanced sensitivity to non-noxious and noxious stimuli (Vanderah et al., 2000; Gardel et al., 2002). Such increased "pain" may manifest behaviorally as a decrease in spinal antinociceptive potency of cannabinoids. Moreover, sustained cannabinoid agonist treatment augments CGRP release in a PKA-dependent manner which may be involved in cannabinoid tolerance (Tumati et al., 2009). Moreover, several kinases such as ERK, PLA, PKC and MAPK have been implicated in the development and maintenance of cannabinoid tolerance (Tappe-Theodor et al., 2007). Other key mechanism involved in analgesics tolerance is overproduction of nitrite and formation of peroxynitrite (Drel et al., 2010). Salvemini et al (2009) demonstrated modulation of
antinociceptive tolerance of cannabinoids and opioids by inhibiting or neutralizing peroxynitrite in diabetic animals.

### 3.5.3 Glial cell and Analgesic Tolerance:

Accumulating line of evidence indicates the prominent role of glial cells in the development and maintenance of NP and has evolved over the past decade (Ren et al., 2008b). It is well known that both microglia and astrocyte are activated following nerve injury in the spinal cord either peripheral nervous system (PNS) or central nervous system (CNS) (Miller, 2005). Glial cell activation has also been implicated in post-traumatic models, inflammatory models, central demyelinating disorders (Watkins and Maie, 2003; Scholz and Woolf, 2007), and in diabetes and its complications (Tsuda et al., 2008; Wodarski et al., 2009). Moreover, it has been suggested that glial cells in the spinal cord play an important role in facilitation of pain, associated with profound morphological changes in microglia and participate in neuronal hypersensitivity including diabetes induced hyperalgesia and allodynia (Daulhac et al., 2006; Talbot et al., 2010).

Astrocyte and glial inhibitors or glia modifying drugs such as fluorocitrate, propentofylline and minocycline have been demonstrated to modify pain sensitivity (Sweitzer et al., 2001; Hua et al., 2005). Microglia cells are regarded as a main source of bioactive endogeneous mediators, including pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα and NO (Hanisch, 2002). Activated microglia following nerve injury in spinal cord increased expression of P2 purinoceptors, mainly P2X4 and P2X7 as well as P2Y2, P2Y6, and P2Y12 (Inoue, 2006a & 2008 b; Ulmann et al., 2008). It was shown in an animal model of neuropathic pain that microglial P2X4 and P2X7 receptors are crucial in pain signaling after peripheral nerve lesion (Chessell et
The ATP receptor P2X4 is up-regulated in spinal cord microglial cells after peripheral nerve injury, and intrathecal injections of P2X4 antisense oligodeoxynucleotides attenuate nerve injury-induced tactile allodynia. Consistently, nerve injury-induced tactile allodynia is also markedly attenuated in P2X4 knockout mice (Tsuda et al., 2009).

In addition, activation of glial cells in spinal cord has also been implicated in morphine and cannabinoids analgesic tolerance (Watkins et al., 2005; Zhou et al., 2010). Inhibition of P2X4 receptors attenuates morphine tolerance, Iba1, glial fibralry acidic protein (GFAP—a marker of astrocyte) and opioid receptor protein expression while enhancing perivascular microglial ED2 (Horvath et al., 2010). Together, these lines of evidence point to a potential role of glial cells and purinoceptor including P2X4 and P2X7R in the development of morphine tolerance (Clark et al., 2010; Zhou et al., 2010). Pre-emptive treatment or co-administration of minocycline and pentoxypylline/propentoxifylline with morphine prevent the development and reversed established morphine tolerance in mice (Ledeboer et al., 2007; Ragavendra et al., 2004).

Another important microglial system is the family of cannabinoid (CB) receptors and its endogenous ligands. Endocannabinoids modulate microglial cell migration without disturbing their ability to phagocytose particles or produce nitric oxide. Although endocannabinoids, including anandamide and 2-arachidonoylglycerol (2-AG), act upon CB1 and CB2 receptors are secreted by neurons, they are more prominently produced in microglial cells during neuro-inflammatory conditions (Romero-Sandoval et al., 2008). Although both CB1 and CB2 receptors are expressed in activated microglial cells, their cellular expression is different, with CB2 receptors abundantly expressed at the leading edges of activated microglia that are implicated in DPN.
Activation of microglia enhances the release of pro-inflammatory cytokines and nitric oxide that are implicated in neuronal hypersensitivity (Correa et al., 2009). Increased level of cytokines induced nitric oxide has been implicated on the development of cannabinoid tolerance (Spina et al., 1998; Banafshe et al., 2005). Therefore, glial cell activation following nerve injury may be involved in development of cannabinoid tolerance. In vivo studies have demonstrated that stimulation of CB2 receptors reduces microglial activation and the expression of pro-inflammatory cytokines in models of neuroinflammation hypoxia-ischemia and Huntington’s disease (Correa et al., 2010b). Moreover, spinal microglial and perivascular cell cannabinoid receptor type 2 activation reduces behavioural hypersensitivity without tolerance after peripheral nerve injury (Romero-Sandoval et al., 2008). These studies indicate potential role of CB2 receptor in modulation of neuronal hypersensitivity via suppression of glial cell mediated neuronal toxicity.

3.5.4 Protein Kinases and Analgesic Tolerance: Hyperglycemia induced activation of PKC is known to contribute in PDN (Evcimen and King, 2007). PKC-mediated phosphorylation of the NMDA receptor expels the Mg\(^{2+}\) ion that at rest blocks the Ca\(^{2+}\) channel (Chen and Huang, 1992). The unblocked channel no longer requires depolarization for activation, resulting in a positive feedback loop of amplified NMDA-receptor responses and further activation of PKC (Fan et al., 1998; Martin et al., 2001). Also phosphorylation of the \(\mu\)-opioid receptor by PKC may uncouple the receptor from its G-protein or alter the properties of its associated potassium channel. In either case, the result is reduced responsiveness of the \(\mu\)-opioid receptor to exogenous opioid drugs, i.e., tolerance. Interestingly, a recent study has shown that activation of PKC decreases CB1 receptor and \(\mu\)-opioid-receptor mRNA levels, suggesting that PKC also inhibits CB1 and \(\mu\)-opioid-receptor turnover (Garcia et al.,
The relationship between this effect and opioids or cannabinoids tolerance remains to be determined.

Administration of the GM1 ganglioside blocks both the translocation of PKC from cytosol to membrane and the development of analgesic tolerance to morphine (Mayer et al., 1995). Additionally, GM1 ganglioside prevents the development of thermal hyperalgesia associated with morphine tolerance (Mao et al., 1992).

PKC is not the only enzyme capable of phosphorylation and desensitization of μ-opioid receptors. Two G-protein-coupled receptor kinases, β-adrenergic receptor kinase 2 and β-arrestin; also synergistically desensitize μ-opioid receptors (Bohn et al., 2000). Furthermore, knockout mice that lack β-arrestin 2 display neither μ-opioid-receptor desensitization nor tolerance to the antinociceptive effects of chronically administered morphine (Przewlocka et al., 2002; Bohn et al., 2002; Raehal et al., 2005). The relationships between the latter two mechanisms and PKC remain to be explored.

Selective inhibitors of PKC-β such as ruboxistaurin ameliorated several neuropathic deficits in experimental diabetic neuropathy (Carolina et al., 2007a; Danis and Sheetz, 2009). In a Phase II-clinical trial, ruboxistaurin at a dose of doses of 32-64 mg day\(^{-1}\) attenuated neuropathic symptoms and deficits, including overall neurologic examination and patient global assessment (Carolina et al., 2007b). In addition to this, PKC may act directly on the CB1 receptor and/or downstream from the receptor (Wallace et al., 2009). Moreover, PKA, Src kinase and MAPK have also been demonstrated to be involved in the development of opioids (Chen et al., 2009) and THC tolerance in mice (Rubino et al., 2005).
3.5.5 **Excitatory amino acid and analgesics tolerance:** Excitatory amino acid (EAAs) such as glutamate and aspartate are the principal excitatory neurotransmitters in neuronal circuits and are involved in a variety of central nervous system functions, including neuropathic pain (Fundytus *et al.*, 2001; Zhang *et al.*, 2009). Glutamate mediates its actions through two types of receptor, metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). Metabotropic glutamate receptors form a family of eight subtypes (mGluR1-8), subdivided into three groups (I-III) that initiate their biological effects by G protein-linked intracellular signal transduction. Their expression throughout the mammalian nervous system implicates these receptors as essential mediators of a cell's fate during injury to the nervous system. Activation of group-II (mGluR2 and -3) or group-III metabotropic glutamate receptors (mGluR4, -6, -7 and -8) has been established to be neuroprotective *in vitro* and *in vivo* (Osikowicz *et al.*, 2008). By contrast, group-I mGluRs (mGluR1 and -5) need to be antagonized in order to evoke protection.

Numerous studies have shown that mGluR 5 antagonists inhibited inflammatory and neuropathic pain in rats and mice (Sotgiu *et al.*, 2003; Li *et al.*, 2010). In addition, acute and chronic administration of mGluR 1 and mGluR 5 antagonist also attenuates STZ induced allodynia and hyperalgesia (Li *et al.*, 2010). These findings suggest that diabetes-induced hyperalgesia and allodynia may be the consequence of increased activity of primary afferent fibres leading to an increased excitatory tone within the spinal cord. An increased glutamatergic input and N-methyl-D-aspartate (NMDA) receptor activity has been demonstrated to contribute to central sensitization in PDN (Chen and Pan, 2003; Wang *et al.*, 2007e), and would maintain the hyperalgesic state (Larsson and Broman, 2010). Moreover, intrathecal morphine challenge induced activation of spinal mGluRs plays a crucial role in the development of tolerance to the
analgesic effects of morphine (Chen et al., 2009; Li et al., 2010) and cannabinoids (Lee et al., 2005). Further, knockdown of spinal metabotropic glutamate receptor 1 (mGluR (1)/5 alleviates pain and restores opioid efficacy after nerve injury in rats (Fundytus et al., 2001). Co-administration of morphine with an NMDA antagonist such as ketamine, memantine and MK-801 or NMDA receptor/glycine site antagonist (ACEA-1328) not only inhibited morphine tolerance development, but also blocked morphine-induced spinal EAAs release (Adam et al., 2008; Chen et al., 2009).

Ionotropic glutamate receptors are ligand-gated nonselective cation channels which allow the flow of K⁺, Na⁺ and sometimes Ca²⁺ in response to glutamate binding. Upon binding, the agonist will stimulate direct action of the central pore of the receptor, an ion channel, allowing ion flow and causing EPSC (excitatory post-synaptic current). NMDA receptors have an internal binding site for an Mg²⁺ ion creating a voltage dependant block which is removed by outward flow of positive current. Since the block must be removed by outward current flow, NMDA receptors rely on the EPSC produced by AMPA receptors to open. NMDA receptors are permeable to Ca²⁺ which is an important cation in the nervous system and has been linked to gene regulation (Morgado et al., 2009; Gover et al., 2009). Accumulating evidence suggested that the flow of Ca²⁺ through NMDA receptors can cause both LTP and LTD by transducing signaling cascades and regulating gene expression (Huber et al., 1995).

Interaction between NMDA and PG receptor-mediated events during inflammatory nociception has also been reported (Buritova et al., 1996). PGE2 was shown to stimulate the release of NO from rat spinal cord by NMDA receptor activation through EP1 receptors. Moreover, cross-communication between the NMDA mediated NOS and COX systems has also been demonstrated (Tetsuka et al., 1994).
Thus, cyclooxygenase-2 inhibitor which when administered by itself or in combination with the nontoxic NMDA receptor antagonist and/or substance that blocks a major intracellular consequence of NMDA receptor activation provides significant analgesic activity (Price et al., 1996; Buritova et al., 1996). Moreover, combination of ketamine (NMDA receptor channel blocker) with ketoprofen (cox inhibitor) produced synergistic depression of NMDA mediated transmission in spinal cord of neonatal rats (Lizarraga et al., 2008). These finding indicates that glutamate mediated activation of NMDA receptor has a pivotal role in the spinal cord in the establishment and maintenance of inflammatory pain hypersensitivity reaction in diabetic patients.

### 3.5.6 MAPK and Analgesic Tolerance:

Recent studies have reported that MAPKs are activated in experimental neuropathic pain models, including PDN (Purves et al., 2001; Sweitzer et al., 2004). All three groups of MAPK have shown to be activated by osmotic perturbations derived from glucose itself or from the polyol pathway (Cohen, 1997; Kultz and Burg, 1998), by oxidative stress (Wang et al., 1998b), and by advanced glycation end products (AGE) via the receptors for AGE –RAGE (Thornalley, 1998). Moreover, peripheral nerve injury or axotomy has been shown to induce long-term JNK (JNKs, also called stress activated protein kinases, SAPKs) activation in dorsal root ganglion (DRG) neurons (Kenney and Kocsis, 1998; Mielke and Herdegen, 2000). After peripheral nerve damage, phosphorylation of both p38 MAPK and JNK were observed in DRG and glial cells (i.e., microglia and astrocytes) of the spinal cord and are associated to the painful behaviour generated in these models, including mechanical allodynia (Sweitzer et al., 2004; Cheng et al., 2010). Treatment with each inhibitor of p38 MAPK and JNK as well as ERK attenuated the magnitude of neuropathic pain (Zhuang et al., 2006).
Long-term treatment of STZ-induced diabetic rats with a p38 inhibitor and selective MAPK/ERK-kinase (MEK) inhibitor PD 198306 prevents neuronal dysfunction such as the archetypal defect of slowed nerve conduction (Agthong and Tomlinson, 2002; Price et al., 2004) and blocked allodynia and hyperalgesia (Sweitzer et al., 2004; Cheng et al., 2010). Moreover, inhibition of p38 mitogen-activated protein kinase attenuates cytokines induced thermal hyperalgesia and inducible nitric oxide synthase expression in the spinal cord (Sung et al., 2005). There is substantial evidence indicating that MAPKs can be activated by chronic morphine treatment in spinal dorsal horn, cultured DRG neuron, as well as in DRG neurons taken from tolerant animals (Chen and Sommer, 2009). MAPKs inhibitors have been reported to attenuate morphine tolerance in various experimental DN in rats and mice (Cui et al., 2006; Chen and Sommer, 2009; Wang et al., 2009f).

3.5.7 Nitric Oxide and Peroxynitrite (ONOO-) and Analgesic Tolerance:

Hyperglycaemia stimulates the production of AGEs, activates PKC, and enhances the polyol pathway leading to increased superoxide anion formation, which plays an important role in the development of PDN (Johansen et al., 2005; Pazdro and Burgess, 2010). Hyperglycaemia also favours the generation of NO, through the activation of NF-κB and increased expression of iNOS, which may increase the generation of cytokines and NO (Cameron et al., 2008; Afanasev et al., 2010). Superoxide anion interacts with nitric oxide, forming the powerful oxidant peroxynitrite (ONOO−) and the generated cytotoxin peroxynitrite leading to PDN (Obrosova et al., 2004; Zheng et al., 2009; Negi et al., 2010) via multiple mechanisms (Fig-2).

Increased NO and nitrosative formation has been documented in vascular endothelium (Pacher et al., 2005), myocardium (Pacher et al., 2005), retina (Obrosova et al., 2005), and kidneys (Thuraisingham et al., 2000; Drel et al., 2006b) of STZ-diabetic animals
and human subjects with diabetes (Afanasev et al., 2010). Moreover, an increased nitrotyrosine immunoreactivity has also been demonstrated in peripheral nervous system i.e., peripheral nerve, spinal cord and dorsal root ganglion (DRG) neurons of STZ-diabetic rats (Cheng and Zochodne, 2003; Obrosova et al., 2005b). Moreover, some studies implicated peroxynitrite in motor and sensory nerve conduction deficits, thermal hypoalgesia, and impaired nitrergic innervation, in STZ-diabetic rats and mice (Pavlov et al., 2007). These neuropathic pain behaviours symptoms of STZ-diabetic rats and mice were normalized by peroxynitrite decomposition catalyst (Matthew et al., 2004; Obrosova et al., 2004; Salvemini and Neumann, 2010).

In addition, NO and peroxynitrite has been implicated in the development of antinociceptive tolerance (Joharchi and Jorjani, 2007; Muscoli et al., 2007; Salvemini, 2009; Salvemini and Neumann, 2010). Considerable evidence supports the notion that a key biologically relevant feature of peroxynitrite is post-translational tyrosine nitration and consequent modification of protein function involved in pain and antinociceptive tolerance (Yamakura et al., 1998). A key example of lost enzyme activity due to nitration in vivo is mitochondrial that normally keeps concentrations of superoxide under tight control (Radi, 2004; Yamakura et al., 1998: Tangpong et al., 2008).

It has been demonstrated that repeated administration of morphine leads to spinal nitration and enzymatic inactivation of MnSOD and that inhibition of peroxynitrite blocks nitration, restores the enzymatic activity of the enzyme and blocks tolerance suggesting the key role of nitrated MnSOD as a source of peroxynitrite in tolerance (Muscoli et al., 2007; Doyle et al., 2009). Moreover, opioids prime the release of pro-inflammatory cytokines and TNF-α mediated nitric oxide production enhances manganese superoxide dismutase nitration and mitochondrial dysfunction in primary
neurons (Tangpong et al., 2008). Inhibition of iNOS or removal of O2- blocked these biochemical changes and inhibited the development of tolerance (Arora et al., 2008), pointing to peroxynitrite, the product of the interaction between O2- and NO, as a signaling mediator in this setting (Doyle et al., 2009; Drel et al., 2007; Drel et al., 2010). Co-administration or combination of morphine with the ONOO- decomposition catalyst, Fe(III) 5,10,15,20-tetrakis (N-methylpyridinium-4-yl)porphyrin, blocked protein nitration, attenuated the observed biochemical changes, and prevented the development of tolerance in a dose-dependent manner (Drel et al., 2007; Salvemini, 2009; Salvemini and Neumann, 2010). Collectively, these data suggest a crucial role for ONOO- in pathways culminating in the development of tolerance to opioids, cannabinoids, NSAIDs and modulators effect on neurosteroids (Figure: 2).

**Fig-02**: Possible involvement of peroxynitrite (ONOO-) in PDN.

Despite the potential role of NO and ONOO in neuropathy, very few study examined
and study the detrimental effect of NO and the ONOO decomposition catalyst mediated protection against neuronal injury. Thus, it was proposed that therapeutic manipulation of nitric oxide and of peroxynitrite may be inhibited STZ-induced neuropathic pain behaviours and analgesics tolerance.