SECTION 4:

REVIEW OF LITERATURE
Drug dependence has been defined by the WHO expert committee as “A cluster of physiological, behavioural and cognitive phenomena of variable intensity, in which the use of a psychoactive substance(s) takes on a high priority. The necessary descriptive characteristics are preoccupation with a desire to obtain and take the drug and persistent drug-seeking behaviour. Determinants and problematic consequences of drug dependence may be biological, psychological or social, and usually interact” (World Health Organization, 1964).

The most common psychoactive substances that are generally misused can be divided into depressants (e.g. alcohol, sedatives/hypnotics, volatile solvents), stimulants (e.g. nicotine, cocaine, amphetamines, ecstasy), opioids (e.g. morphine and heroin), and hallucinogens (e.g. PCP, LSD, cannabis) (World Health Organisation, 2005).

Opioids are standard drugs used to manage severe pain and are the most commonly used psychoactive substances across the world. Opiate drugs which include heroin, morphine, codeine, Oxycontin, Dilaudid and methadone and well known to elicit profound euphoria and mental relaxation in treated subjects. Therefore, opioids are one of the most widely abused drugs in the world. Their chronic use is associated with the development of debilitating form of dependence in subjects (Akil H and Lewis, 1987; Van Ree et al., 1999; Hardman et al., 2001). Opioid dependence is characterized by a cluster of cognitive, behavioural and physiological features. The International Classification of Diseases, 10th edition (World Health Organization, 2007) has identified six such characteristic features of opioid dependence: a strong desire or sense of compulsion to take opioids; difficulties in controlling opioid use; a physiological withdrawal state; tolerance; progressive neglect of alternative pleasures or interests because of opioid use and; persisting with opioid use despite clear evidence of overtly harmful consequences. Opioid dependence is a worldwide health problem that has enormous economic, personal and public health consequences. Systematic studies place
the burden of harm from opiate use in an estimated 15.6 million illicit opioid users in the world (United Nations Office on Drugs and Crime, 2007). Opioids are the main drugs of abuse in Asia, Europe and much of Oceania, and it is estimated that globally the consumption of the opioid class of drugs is increasing (United Nations Office on Drugs and Crime, 2007). Opioid dependence imposes a significant economic burden on society, not only in terms of directly attributable health-care costs (e.g. treatment and prevention services, and other health-care use), but also in terms of its impact on other budgets (notably social welfare and criminal justice services). Opioid dependence also has an effect on productivity, due to unemployment, absenteeism, and premature mortality. Studies in some countries have attempted to place an economic value on the aggregate impact of these consequences, with findings of from 0.2 to 2.0 % of a country’s gross domestic product (Collins and Lapsley, 1996; Xie et al., 1998; Clark et al., 2003).

4.1 Available Clinical Approaches to Treat Opioid Dependence

Pharmacological management of opioid dependence associated opioid withdrawal syndrome is usually done by one of the following methods: detoxification using opioid antagonists (i.e. naltrexone and naloxone); gradual cessation of an opioid agonist (i.e. methadone); short-term use of a partial opioid agonist (i.e. buprenorphine); sudden opioid cessation and; use of drugs that do not work directly on the opioid receptors (alpha-2 adrenergic agonists viz., clonidine and lofexidine) so as to relieve withdrawal symptoms (Hardman et al., 2001; Krantz and Mehler, 2004; Lee et al., 1987). Further, the activation of endogenous opioid system by non-pharmacological methods like acupuncture or transcutaneous electrical stimulation has also been shown to be clinically effective (Hardman et al., 2001).

Detoxification
Detoxification refers to removal of opioid drugs from the body in a controlled and humane fashion while maximizing treatment retention and minimizing degree of discomfort (Amato et al., 2005). An individual undergoing detoxification experiences withdrawal, which includes symptoms of irritability, anxiety, chills, nausea, diarrhea, sweating, sneezing, bone and muscle weakness, and insomnia (Doyon, 2004; Gowing et al., 2008), and although not life-threatening, is difficult for most to endure. Detoxification is a necessary process for initiating long-term abstinence-based treatments, and a prerequisite for admittance into maintenance or other rehabilitation programs.

There are two general approaches to detoxification: abrupt termination of opioid use, potentially precipitated by an opioid antagonist, with administration of an alpha2 adrenergic agonist (e.g., clonidine) to reduce withdrawal symptoms (e.g., Gowing et al., 2008), or gradual tapering of the opioid drug dose followed by buprenorphine or methadone replacement (e.g., Calsyn et al., 2006).

Further, it has been suggested that psychosocial treatments may be used to increase adherence and increase social support variables known to influence positive outcomes (Amato et al., 2008a). For example, contingency management, community reinforcement, psychotherapeutic counselling and family therapy, when added to pharmacotherapy for detoxification, result in higher rates of treatment compliance, treatment completion, number of patients abstinent at follow-up, and lower rates of opioid use compared to pharmacological treatment alone.

**Abstinence-focused long term interventions**

In order to completely eradicate opioid-dependence, abstinence is typically attempted via a slow taper using opioid agonists, as described above, or an initial detoxification phase followed by a relapse prevention phase. The relapse prevention phase is aimed at the long-
term maintenance, facilitated by opioid antagonist naltrexone as it decreases opioid craving (Gonzalez and Brogden, 1988). Adding supplementary treatment components has been shown to increase the effectiveness of naltrexone interventions, with the goal of lowering attrition rates and increasing overall treatment efficacy. Examples include combination buprenorphine/naltrexone treatment (Gerra et al., 2006), inclusion of psychosocial treatments (Nunes et al., 2006), and incorporating families into the treatment process (Fals-Stewart and O'Farrell, 2003).

The principal goals of maintenance are to reduce craving for opioids, lessen negative withdrawal symptoms, and block the euphoric effects from any future use of narcotics by giving opioid addicts a controlled dose of an opioid agonist having lower dependence potential (van de Brink and Haasen, 2006). Treatment is aimed at reducing intensity, frequency, and length of relapse (Leshner, 1998), limiting overdose risk, criminal activity, HIV infection, and promoting psychosocial adjustment (Farrell et al., 1994). Although still physically dependent on the substitution medication, maintenance treatments result in decreased time spent on drug-related activities and may allow dependent individuals to transition into abstinence based programs (Ward et al., 1999). The four most frequently studied medications for maintenance treatment are methadone, Levacetylmethadol (LAAM), buprenorphine, and heroin itself (diacetylmorphine).

Methadone

Methadone, a full-opioid agonist that has been in use since the 1960s (Dole and Nyswander, 1965), is the most well-studied and utilized drug for treatment of opioid dependence (O'Connor, 2005; van de Brink and Haasen, 2006), as methadone is significantly safer than heroin (Pond et al., 1985).

Buprenorphine
Although methadone maintenance has been established as an effective treatment for opioid addiction, it is associated with a number of problems, such as high attrition rates within the first month (Maxwell and Shinderman, 2002), and reliance on methadone clinics, which are required because methadone must be administered daily. Moreover, being a full-opioid agonist, methadone itself is susceptible to abuse and overdose, including adverse reactions such as respiratory depression. For these reasons, buprenorphine, a partial agonist with a ceiling effect for respiratory depression (Ling and Compton, 2005), may be a safer intervention. When compared to placebo, buprenorphine at medium and high doses improved treatment retention and opioid use as assessed by urinalysis (Schottenfeld et al., 2008).

Levacetylmethadol (LAAM)

LAAM, another full-opioid agonist, is associated with greater suppression of heroin use compared to methadone (Clark et al., 2002). However, LAAM participants drop out of treatment at a higher frequency than methadone patients, likely due to increased side effects. Moreover, the safety profile of LAAM has been called into question through the possibility of “torsade de pointes,” a potentially fatal ventricular arrhythmia (Clark et al., 2002; Wedham et al., 2007; Anglin et al., 2009).

Heroin (diacetylmorphine)

Oviedo-Joekes et al. (2009) compared methadone to diacetylmorphine for patients with a history of unsuccessful agonist treatment. They found that compared to methadone, diacetylmorphine increased treatment retention and reduced engagement in illegal activities (including heroin use). Similarly, a group in Germany (Haasen et al., 2007) randomized patients to either heroin-assisted maintenance or methadone, and found that the heroin-assisted patients stayed in treatment longer, showed better health outcomes and exhibited less illicit drug use. However, the latter trial found increased adverse effects in the heroin group.
(respiratory depression and seizure). For these reasons, heroin-assisted maintenance should be used with caution, although it may be an option for patients with a failed history of traditional agonist treatment (Haasen et al., 2007).

Psychosocial approaches

Psychosocial treatments are also important avenues for improving relapse and treatment retention. However, insufficient clinical evidence exists to support psychosocial treatments without medication or even along with agonist treatment (Mayet et al., 2004; Amato et al., 2008b).

Limitations in the Present Clinical Approaches

The above treatment of opioid dependence focuses on prevention of the development of dependence, elimination of existing dependence and suppression of symptoms associated with drug withdrawal (Jaffe, 1987). These pharmacotherapies target specific neurotransmitter systems, which have been shown to mediate the acute and chronic effects of opioids. Moreover, opioid antagonists and agonists are used in the treatment of opioid abuse despite the fact that some therapies (e.g., naltrexone) are not only ineffective, but, in addition, are consistently refused by addicted individuals. In opioid substitution therapy (e.g., with methadone), Ball and Ross (1991) reported a recidivism rate after discontinuation of treatment of 80%. Unfortunately, the existent efficacy data on above treatment options is therefore not encouraging (Minozzi et al., 2006). It is also important to note that the rates of relapse in various studies are on a relatively higher side, because many patients are unable to endure withdrawal syndrome (Williams et al., 2001; van de Brink and Haasen, 2006). Moreover, there is evidence suggesting that low compliance associated with above pharmacological interventions can actually have deleterious long-term consequences viz., return to a fully opioid dependent lifestyle (Sullivan et al., 2006). Therefore, questions
regarding the absolute efficacy of the above pharmacological approaches remain, and are thus likely to fuel further research into the identification of novel therapeutic targets which might more effectively modulate the pathogenesis of opioid withdrawal syndrome. Moreover, development of newer pharmacological interventions which are able to attenuate the progression of opioid dependence based precipitation of opioid withdrawal syndrome are proposed to facilitate the possibility of a more effective treatment procedure, lower propensity to relapse and a better quality of life.

4.2 Pathophysiology of Opioid Withdrawal Syndrome

Principal sites of brain involved in the expression of morphine withdrawal syndrome

The locus coeruleus (LC), a nucleus in the anterior pons, is the principal site in brain that triggers the onset of opioid withdrawal syndrome (Maldonado et al., 1992). Neurons present in LC possess a high density of µ-opioid receptors and are noradrenergic in nature. LC region represents the primary source of noradrenergic innervation of the limbic system and the cerebral and cerebellar cortices (DahlstroÈm and Fuxe, 1964; Moore and Bloom, 1979; Foote et al., 1983). The opioid receptor linked mechanism based NAergic activity in the LC neurons is known to be causative of opioid withdrawal syndrome (Aghajanian & Wang, 1987).

Moreover, it has been documented that the periaqueductal gray matter and nucleus raphe magnus are involved in the expression of various somatic components of morphine withdrawal syndrome (Maldonado et al., 1992). Furthermore, the motivational and affective aspects of morphine withdrawal, as demonstrated by the aversive stimulus effects or negative reinforcing effects, involve certain elements of nucleus accumbens (Maldonado et al., 1992; 1996).
Neurobiology of opioid withdrawal syndrome

Opioid dependence and some of the most debilitating opioid withdrawal symptoms originate from changes in an important brain system, involving an area at the base of the brain—the locus ceruleus (LC) (Maldonado et al., 1992). Neurons in the LC produce a chemical, noradrenaline (NA), and distribute it to other parts of the brain where it stimulates wakefulness, breathing, blood pressure, and general alertness, among other functions. When opioid molecules link to µ receptors on brain cells in the LC, they suppress the neurons’ ability to produce cAMP and resultantly decrease the release of NA, resulting in drowsiness, slowed respiration, low blood pressure—familiar effects of opioid intoxication. With repeated exposure to opioids, however, the LC neurons adjust by increasing their level of activity. Now, when opioids are present, their suppressive impact is offset by this heightened activity, with the result that roughly normal amounts of NA are released and the patient feels more or less normal. When opioids are not present to suppress the LC brain cells’ enhanced activity, however, the neurons release excessive amounts of NA, triggering jitters, anxiety, muscle cramps, and diarrhea (DahlstroÈm and Fuxe, 1964; Moore and Bloom, 1979; Foote et al., 1983).

Other brain areas in addition to the LC additionally contribute to the production of withdrawal symptoms, including the mesolimbic reward system. For example, opioid tolerance that reduces the Ventral tegmental area’s (VTA) release of dopamine (DA) into the Nucleus accumbens (NAc) prevents the subject from obtaining pleasure from normally rewarding activities such as eating. These changes in the VTA and the DA reward systems, though not fully understood, form an important brain system underlying craving and compulsive drug use (Kreek and Koob, 1998).

Biochemical pathway involved in the precipitation of opioid withdrawal syndrome

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Opioid receptor signaling is transmitted to intracellular effectors through G transducer proteins from different subfamilies (Gαo, Gaι1-3, Gaε and Gaq/11). Several lines of evidence indicate that opioid receptors predominantly couple to pertussis toxin sensitive Gaι and Gao classes which are responsible for the inhibition of adenylyl cyclase (AC; Subtype I & VII) /cyclic adenosine mono-phosphate (cAMP) pathway which decreases the firing rate of LC neurons. However, sustained opioid receptor activation in the locus coeruleus neurons has been shown to cause the uncoupling of opioid receptors from their Gαo and Gaι proteins and linked effector pathways (Liu and Anand, 2001). This uncoupling of opioid receptors to the pertussis toxin sensitive Ga inhibitory subunits causes the reduction of the inhibitory effect of opioids on AC activity, eventually resulting in a manifold up-regulation of the AC/cAMP pathway above normal levels (Liu and Anand, 2001; Nestler, 2004). Moreover, opioid receptor linked pertussis toxin resistant Gaε and Gaq/11 protein associated βγ dimers have been proposed to enhance the inhibitory response of adenylyl cyclase to opioid agonists (Mostany et al., 2008). Some reports in the literature have described a switch from Gaι to Gaε proteins in opioid-receptor G-protein coupling following the formation of µ-δ heterodimeric receptors resulting in further enhancement of AC activity (George et al., 2000; Fan et al., 2005). This compensatory increase in AC activity in locus coeruleus and resultant noradrenergic transmission of impulses to various parts of the brain is associated with the development of physical dependence in terms of opioid withdrawal syndrome (Sharma et al., 1977; Law et al., 1982; Koob and Bloom, 1988; Liu and Anand, 2001; Nestler, 2004; Pineyro and Archer-Lahlou, 2007). Similar neuronal activation is also observed in the ventral noradrenergic neurons projecting to the bed nucleus of the stria terminalis and are proposed to contribute towards the causation of opioid withdrawal-induced aversion (Delfs et al., 2000).

However, till date, the precise mechanism regulating the adenylyl cyclase superactivation
during chronic exposure, which causes the appearance of opioid withdrawal syndrome remains unclear. A number of biochemical processes have been proposed to mediate the above molecular events. Altered protein phosphorylation of adenylyl cyclase isoforms is hypothesized to underlie persistent opioid receptor activation related AC super-activation (Chakrabarti et al., 1998a; 1998b). Tyrosine kinases, protein kinase-C and raf-1 have been reported to participate in this phosphorylation of adenylyl cyclase isoforms (Varga et al., 2003). Further, experimental data has shown that phosphorylation based coupling of opioid receptor to Gs proteins (Crain and Shen, 1996; Tso and Wong, 2001), Gβγ subunits (Avidor-Reiss et al., 1996), or the increase in the constitutive receptor activity (Chavkin et al., 2001; Liu et al., 2001b) might be considered as a mechanistic basis for AC super-activation. Moreover, evidence shows that opioid receptor phosphorylation upon protracted agonist stimulation plays a critical role in these processes. Protein kinases proposed to be responsible for opioid receptor phosphorylation have been reported to include protein kinase C, cAMP dependent protein kinase, G-protein-coupled receptor kinase and Calcium (Ca2+)/calmodulin-dependent protein kinase (CaMK II) (Yu, 1996; Nestler and Aghajanian, 1997; Lu et al., 2000b).

Recently, AC superactivation has been demonstrated to be dependent on the receptor localization in membrane microdomains known as lipid rafts/caveolae (Zhao et al., 2006), which are enriched with a variety of signalling proteins (Lisanti et al., 1994). This finding further indicates that the colocalization of opioid receptors, β-arrestins, protein kinases and AC subtypes in lipid rafts/caveolae might also be an important step for the regulation of receptor signaling and trafficking during long-term agonist treatment.

Moreover, chronic activation of opioid receptors has been shown to recruit activated Src to form a complex with the opioid receptors and Gαi2 protein. The opioid receptor-Gαi2-Src
signaling complex causes the phosphorylation of tyrosine residues present on Src-kinase and thus activates it, further enhancing the conversion of opioid receptor into the receptor tyrosine kinase like signaling complex. This opioid receptor-Gα_{i2}-Src-Src-kinase complex, either directly or indirectly, by activating other protein kinases, phosphorylates AC isoforms, such as AC5/6 and other signaling molecules, which eventually leads to the observed the AC super-activation noted during opiate withdrawal (Avidor-Reiss et al., 1996; Avidor-Reiss et al., 1997; Chakrabarti et al., 2001; Zhang et al., 2009).

Investigations have shown that adenylyl cyclases also are intimately associated with sites of calcium ion entry into the cell. Moreover, variations in cellular cyclic AMP levels are predicted to arise because of feedback inhibition of adenylyl cyclase by Ca^{2+} (Cooper et al., 1995). Further, AC activation has been reported to be associated with the activity of calcium and calcium dependent proteins viz., protein kinase C (PKC), calmodulin, calcineurin, Gia, Gsa and Gβ/γ (Mons and Cooper, 1995; Mons et al., 1995; Homayoun et al., 2003; Li et al., 2006). Moreover, chronic opioid receptor activation linked AC super-activation, which is particularly accentuated during opioid withdrawal, is proposed to stem from this activation of calcium and calcium dependent proteins viz., protein kinase C (PKC), calmodulin, calcineurin, Gia, Gsa and Gβ/γ (Mons and Cooper, 1995; Mons et al., 1995; Homayoun et al., 2003; Li et al., 2006).

The adenylyl cyclase super-activation based increase in cytosolic cAMP levels is noted to trigger the release of substantial amounts of noradrenaline from the neurons present in the locus coeruleus by a calcium sensitive pathway. Noradrenaline, thus released, causes the emanation of a multitude of impulses from locus coeruleus to different areas of the brain, where it leads to the excitation of various parts of the brain involved in the regulation of different physiological events. The resultant aberrations in such physiological events are
considered as the characteristic hallmarks of opioid withdrawal syndrome (Sharma et al., 1977; Williams et al., 2001). Investigations into biochemical mechanisms mediating the development of opioid withdrawal syndrome have resulted in identification of a few novel investigational interventions showing promise atleast during preclinical evaluation (Figure 1).
Figure 1: Represents the changes in respective intracellular regulatory systems of transmitter release from nerve terminals are changed after acute and chronic opioid treatment. Left: when applied acutely, opioids inhibit transmitter release by several potential mechanisms. These mechanisms include the activation of potassium conductance, inhibition of calcium conductance. Right: after withdrawal from chronic treatment with morphine, the inhibition of transmitter release by opioids is changed in several ways. 1) Opioids no longer activate voltage-dependent potassium currents to inhibit release. 2) There is an upregulation of adenylyl cyclase that increases transmitter release by activation of PKA. 3) The upregulated adenylyl cyclase is sensitive to inhibition by opioids and represents a new, morphine-induced effector. 4) The increased adenylyl cyclase activity increases the production of cAMP that is metabolized to adenosine such that adenosine tone and thus presynaptic inhibition mediated by A1 adenosine receptors is enhanced at some synapses.
4.3 Targets for Potential Opioid Withdrawal Syndrome Management

4.3.1 Calcitonin gene related peptide (CGRP)

CGRP, a 37-amino acid peptide, is abundantly expressed in high threshold sensory afferent fibers in the central nervous system (Gibson et al., 1984). The role of CGRP as a pronociceptive transmitter is well established and it is noted to be released centrally from nociceptive fibers in response to noxious stimuli (Cridland and Henry, 1989; Morton and Hutchison, 1989; Biella et al., 1991; Yu et al., 1994). Its expression is markedly elevated during inflammation (Donaldson et al., 1992; Mapp et al., 1993; Galeazza et al., 1995; Menard et al., 1995a; 1995b). Studies in cultured adult dorsal root ganglion neurons suggest that opioid-induced increase in CGRP occurs through the activation of mu-, delta-, or kappa-opioid receptors via a protein kinase C-dependent signalling pathway (Ma et al., 2000; Belanger et al., 2002). Upregulation of this peptide is also noted to be mediated by activity of the mitogen-activated protein kinase (MAPK) pathway and involves phosphorylation of cyclic AMP response element binding protein (CREB), a transcription factor regulating CGRP gene expression (Ma et al., 2001). Thus, chronic morphine exposure has shown to initiate and enhance CGRP gene transcription leading to increased cellular peptide levels. Evidence further shows that CGRP activity plays a significant role in the development of opioid physical dependence. Studies in the rat brain have shown that CGRP levels are markedly elevated in the medulla oblongata (Tiong et al., 1992) and in the corpus striatum (Welch et al., 1992) during the antagonist precipitated opioid withdrawal. In morphine dependent animals, an opioid receptor antagonist challenge causes the release of CGRP from neuronal stores is thus noted to precipitate autonomic and somatic signs of opioid withdrawal. Administration of CGRP 8–37, a selective CGRP receptor antagonist, reduces the depletion of the peptide and partially suppresses opioid withdrawal syndrome (Trang et al., 2002),
suggested that activation of CGRP receptors by the peptide released from primary afferents contributes to expression of the morphine withdrawal response. CGRP receptors are G-protein coupled via a unique receptor component protein (RCP) and their activation is noted to increase the intracellular levels of cyclic AMP and thus mediates the genesis of opioid dependence related withdrawal syndrome (Nestler, 1996; Lane-Ladd et al., 1997). This CGRP-related stimulation of cyclic AMP causes the induction of immediate early gene c-fos, and the expression of its protein product Fos during opioid withdrawal (Hass et al., 1991; Reddington et al., 1995; Prillar et al., 1995; 1998a; 1998b).

CGRP in sensory afferents is co-localized with L-glutamate and substance P (Gibbins et al., 1987; Gibbins and Morris, 1987; Merighi et al., 1991), and there is functional interaction between these transmitters, it has been proposed that CGRP acts in concert with these transmitters to contribute to the induction of opioid dependence. CGRP promotes release of L-glutamate (Kangrba et al., 1990) and augments NMDA receptor-dependent depolarization of neurons which in turn leads to the development of physical dependence (Murase et al., 1989; Dunbar and Yaksh, 1996; Jhamandas et al., 1996). Similarly, CGRP is noted to facilitate the release of substance P (Oku et al., 1987). Seybold et al (2003) have demonstrated that CGRP increases expression of both substance P receptor as well as neurokinin (NK)-1. Thus, CGRP may contribute to the development of opioid tolerance and physical dependence partly by influencing the levels of substance P and the activity of NK-1 receptors.

4.3.2 Arachidonic acid metabolites

Prostaglandins (PG)

Prostaglandins are biochemical substances that are generated from arachidonic acid via the
enzymatic activity of cyclo-oxygenase (COX), COX-1 and COX-2. There is evidence that the activity of prostaglandins contributes to the genesis of opioid physical dependence. Dunbar et al. [2000] have demonstrated that acute intrathecal ibuprofen, a selective COX-1 inhibitor, attenuates hyperalgesia associated with morphine withdrawal. Moreover, it has been reported that a number of withdrawal signs, encompassing the sensory, autonomic, and motor components of the opioid withdrawal syndrome, can be partially suppressed by intrathecal administration of COX-2 selective inhibitors, nimesulide and DuP-697 (Trang et al., 2002). These agents additionally decrease the withdrawal-associated release of CGRP in the dorsal horn region, suggesting that a high level of prostaglandin activity may drive neuropeptide release from spinal sensory neurons during morphine withdrawal. Considering that COX-2 is the major isoform constitutively expressed in the central nervous system (Trang et al., 2003) and its expression is increased in response to afferent nociceptive input (Hay and de Bellerache, 1997), an adaptive increase in the expression or activity of this enzyme in response to chronic opioid treatment may contribute to the genesis of dependence. However, since treatment with COX inhibitors only partially suppressed the development of the opioid dependent state, other mediators may also play a role in this phenomenon. Indeed, there is evidence implicating the activity of arachidonate metabolites generated from the lipoxygenase (Capasso and Sorrentino, 1997; Capasso, 1999; Trang et al., 2003; Walters et al., 2003) and endocannabinoid (Ledent et al., 1999; Rubino et al., 2000; Mas-Nieto, 2001) pathways in the induction and expression of opioid dependence.

**Leukotrienes**

Inhibition of COX activity has been shown to shunt arachidonic acid into the 5-, 12-, or 15-lipoxygenase (LOX) pathway, yielding LOX metabolites viz., leukotrienes. Leukotrienes are important bioactive compounds with their role as mediators of multiple inflammatory
processes (Samuelsson, 1983). These metabolites are shown to have a role in the development of physical dependence related opioid withdrawal at the peripheral level (Capasso and Sorrentino, 1997; Capasso, 1999). Moreover, pharmacological inhibition of 5-lipoxygenase, an enzyme mediating the general biosynthesis of leukotrienes, has been observed to block opioid dependence both in vitro using a guinea pig ileum preparation experiment, as well as in vivo in a rodent model of opioid dependence, thus indicating the possible involvement of leukotrienes in the development of opioid withdrawal syndrome (Capasso and Sorrentino, 1997; Trang et al., 2003). Our laboratory has previously shown that selective leukotriene D₄ receptor antagonism attenuates experimental opioid withdrawal syndrome (Rehni et al., 2008a; 2008b). Moreover, we have shown that the inhibition of leukotriene D₄ synthetic pathway exerts an ameliorative effect in a mouse model of naloxone induced opioid withdrawal syndrome (Rehni et al., 2008a). Moreover, effects of LOX inhibitors on opioid dependence are quite similar to those seen previously following intervention with non-selective COX and selective COX-2 inhibitors (Dunbar et al., 2000; Rehni et al., 2008a; 2008b). This suggests that metabolites derived from both pathways likely contribute to development of the opioid dependent state through a common intracellular signalling pathway.

**Endocannabinoids**

Endocannabinoids are a disparate set of neuronal biochemical substances derived from arachidonic acid viz., anandamide and 2-arachidonylglycerol (Egertova et al., 1998). These endocannabinoids are endogenous ligands known to act on the cannabinoid receptors which are classified as CB-1 and CB-2 receptors (Felder et al., 1995). In the nervous system, CB-1 receptors are expressed (Farquhar-Smith et al., 2000) and are noted to be co-localized with mu-opioid receptors and have found to interact with each other (Cichewicz et al., 1999;
Massi et al., 2001; Salio et al., 2001). In CB-1 receptor deficient mice, the severity of the morphine withdrawal response has been noted to be on a lower side (Ledent et al., 1999), indicating the involvement of endocannabinoids in induction of morphine dependence. Reports have also shown that the pharmacological modulation of CB-1 receptors or inhibition of the anandamide reuptake transporter effectively alters the severity of morphine withdrawal response (Rubino et al., 2000; Mas-Nieto et al., 2001; Del et al., 2002). Moreover, evidence that chronic morphine exposure upregulates both CB-1 receptor density and its associated G-protein coupled signalling system points to a potential role for endocannabinoids in development of opioid dependence (Gonzalez et al., 2002; 2003).

4.3.3 N-methyl-D-asparate (NMDA) receptors

The NMDA receptor complex, a ligand-gated cationic channel, is a principal subtype of glutamate receptors which regulate the general excitability of brain (Danysz et al., 1995). Many reports have shown that NMDA receptor modulators attenuate both physical as well as motivational aspects of the expression of morphine dependence when measured in terms of naloxone-precipitated morphine withdrawal syndrome (Cappendijk et al., 1993; Higgins et al., 1992; Popik et al., 1995; Rasmussen et al., 1991; Tanganelli, et al., 1991; Trujillo and Akil, 1991). In addition, NMDA receptor antagonists have been documented to inhibit the development of morphine dependence (Elliott et al., 1994; Tiseo and Inturrisi, 1993; Tiseo et al., 1994; Trujillo and Akil, 1991) as well as its maintenance (Popik and Skolnick, 1996). Moreover, the ameliorative effect of NMDA receptor antagonists (dextromethorphan, ibogaine) has also been reported in preliminary clinical trials (Koyuncuoglu and Saydam, 1990; Lotsof, 1995).
Chronic morphine treatment has been shown to cause the upregulation of NMDA receptors (Lim et al., 2005). During opioid withdrawal, µ-opioid receptor system is noted to activate NMDA receptors, so as to mediate Ca^{2+} influx which subsequently activates various Ca^{2+} dependent second messenger system cascades viz., Ca(2+)/calmodulin kinase II (p-CaMKII) protein pathway. Protracted activation of such Ca^{3+} dependent pathways have been noted to mediate the progression of long term potentiation (LTP) of neurons which thus contributes to the development of opioid dependence (Drdla et al., 2009).

4.3.4 Calcium and Calcium Channels

Studies have shown that changes in neuronal Ca^{2+} levels are involved in mediating opioid dependence (Bhargava, 1978; Harris et al., 1977). Calcium channels are classified pharmacologically into three different types: L-type, T-type, and N-type (Miller, 1987; Nowycky et al., 1985). It has been noted that L-type Ca^{2+} channel plays an important role in the expression of the withdrawal syndrome from opioids (Baeyens et al., 1987). A recent report demonstrates that an increase in the expression of subunits constituting L-type Ca^{2+} channels (α1C, α1D and α2/δ1 subunit) is seen in the cerebral cortex and mesolimbic region. This alteration in the transcriptional production of the calcium channels subunit proteins associated with chronic opioid activation is noted to participate in the development of physical dependence on morphine (Shibasaki et al., 2007). Moreover, functional modifications of Ca^{2+} channels, especially L-type Ca^{2+} channels, in the central nervous system are well recognized as one of the neurochemical events occurring in the development of physical dependence on morphine (Ramkumar and El-Fakahany, 1988). Moreover, it is documented that the pharmacological modulation of L-type Ca^{2+} channels attenuates the
development of opioid withdrawal syndrome in laboratory animals (Tokuyama & Ho, 1996a). This beneficial effect of the calcium channel modulators has been ascribed to their effect on glutamate levels in the locus coeruleus region of brain (Tokuyama & Ho, 1996b).

### 4.3.5 Calcium (Ca^{2+}) / calmodulin-dependent protein kinase

Calcium (Ca^{2+})/ calmodulin-dependent protein kinase (CaMK II) is a multifunctional protein kinase, the activation of which depends on Ca^{2+}/calmodulin, and is noted to be highly concentrated in brain tissues (Hanson & Schulman, 1992). An important characteristic of CaMK II is its autophosphorylation, which is dependent on Ca^{2+}/calmodulin and essential for its activation (Kwiatkowski et al., 1988). Autophosphorylation enables the kinase to phosphorylate substrates in a Ca^{2+}/calmodulin-independent manner and thus prolongs the duration of its effect. Activation of CaMK II in brain has been shown to play an important role in long term potentiation of neurons, a phenomenon known to mediate the development opioid withdrawal syndrome (Hanson and Schulman, 1992; Giese et al., 1998; Drdla et al., 2009). Further, it has been shown that opioid receptor phosphorylation upon agonist stimulation which plays a critical role in the development of opioid dependence is substantially dependent on CaMK II activity (Koch et al., 1997). Moreover, it has been noted that the pharmacological inhibition of CaMK II decreases the severity of morphine withdrawal syndrome in rats (Lu et al., 2000b). Additionally, naloxone-precipitated opiate withdrawal has shown to significantly elevate both the activity as well as transcription of more CaMK II (Lou et al., 1999).

### 4.3.6 Nitric oxide synthase (NOS)
It has been documented that nNOS upregulation takes place during the development of opioid dependence (Cuellar et al., 2000). nNOS inhibition is noted to block the development of morphine dependence (Kolesnikov et al., 1993; Leza et al., 1996). Activation of NMDA receptor gated ion channel, a mechanism known to contribute towards the genesis of opioid dependence, is noted to enhance the influx of $\text{Ca}^{2+}$ into the cells which in turn leads to the activation of calmodulin. This activated calmodulin is further noted to cause the induction of neuronal nitric oxide synthases (nNOS) (Schuman & Madison, 1994). Besides, the GABAergic neurons of the periaqueductal gray (PAG), a site involved in the expression of various somatic components of morphine withdrawal syndrome (Maldonado et al., 1992), have been shown to colocalize to a high extent with nNOS (Lovick & Paul, 1999), further supporting the role of this enzyme in the development of opioid dependence.

### 4.3.7 Gamma aminobutyric acid (GABA)

Gamma aminobutyric acid (GABA) is a principal inhibitory neurotransmitter of the central nervous system and its inhibitory effects are mediated by ionotropic (GABA$_A$ and GABA$_C$) and metabotropic (GABA$_B$) receptors (Enna and McCarson, 2006). Reports have shown that cAMP superactivation, a biochemical hallmark of opioid withdrawal syndrome, acts on a variety of downstream targets of the opioid receptor signalling cascade so as to cause an enhancement of GABA release in the nucleus accumbens and the ventral tegmental area (VTA) of brain (Chieng and Williams, 1998; Shoji et al., 1999). Withdrawal-induced increases in GABA release frequency in the VTA and nucleus accumbens have been shown to correlate with behavioral indices of morphine withdrawal (Chieng and Williams, 1998; Shoji et al., 1999; Madhavan et al., 2010). Such a possibility of vesicular release of GABA during opioid withdrawal syndrome has been ascribed by various groups to the activity of...
voltage-gated calcium channels (Wilding et al., 1995); GIRK currents (Cruz et al., 2008); GABA transporter current that modulate GABA release during morphine withdrawal via a PKA-dependent mechanism in the periaqueductal gray (Bagley et al., 2005); or a cAMP-dependent switch in GABA_A receptor conductance from inhibitory to excitatory that has been reported to occur in rat VTA GABA neurons (Laviolette et al., 2004; Vargas-Perez et al., 2009). Recently, it has been shown that GABA transporter 1 (GAT-1) cation currents directly increase GABAergic neuronal excitability and synaptic GABA release in the periaqueductal gray (PAG) during opioid withdrawal in rodents indicating that dysregulation of transmitter transporter current is an important factor responsible for the maladaptive plasticity that underlies opiate withdrawal (Bagley et al., 2011). Moreover, pharmacological activation of GABA_B receptor in the locus coeruleus region of brain has been shown to attenuate morphine withdrawal syndrome in rats (Riahi et al., 2009).

4.3.8 G-protein-gated inwardly rectifying potassium (GIRK) channels

Opioid receptors are coupled to G-proteins of the G_i/o subfamily, that modulate multiple intracellular effectors, including adenylyl cyclase, voltage-gated Ca^{2+} channels, and G-protein-gated inwardly rectifying K^{+} (GIRK) channels (Williams et al., 2001). GIRK channels mediate in large part the postsynaptic inhibitory effect linked to G_i/o-coupled receptors. Moreover, GIRK channels are widely expressed in the CNS and contribute to GIRK currents in locus ceruleus (LC) neurons (Torrecilla et al., 2002) and dopaminergic neurons of the ventral tegmental area (Labouebe et al., 2007), both of which are in turn involved in the development of opioid dependence. Several studies involving mice with mutant Girk genes have implicated GIRK channels in acute opiate effects (Ikeda et al., 2000;
Mitrovic et al., 2003; Marker et al., 2004). Lately, morphine withdrawal syndrome has been reported to be severely attenuated in GIRK, Kir3 knock-out mice (Cruz et al., 2008). Therefore, GIRK channels have been proposed to constitute an inhibitory gate for the induction of opioid dependence via the postsynaptic inhibition of the adrenergic neurons of the LC (Cruz et al., 2008).

4.3.9 Adenosine

Endogenous adenosine is a potent inhibitory neuromodulator in the central nervous system. Three different subgroups of adenosine receptors exist—A1, A2, and A3—which mediate the differential effects produced by adenosine on various parts of the brain (Palmer and Stiles, 1995). Adenosine has been shown to play a significant role in mediating the pathogenesis of opioid dependence. It has been demonstrated that A1 and A2A adenosine receptors mediate the effect induced by adenosine in opiate withdrawal syndrome and have further shown that adenosine A1 agonists and adenosine A2A antagonists are beneficial in the treatment of this syndrome (Stella et al., 2003). Adenosine induced reduction in the opioid withdrawal symptoms mediated by activating A1 adenosine receptors has been ascribed to its inhibitory effect on excitatory amino acid release as well as negative modulation of intracellular cAMP levels. Moreover, blockade of A2A adenosine receptors has been shown to favourably enhance dopaminergic striatopallidal transmission so as to attenuate the somatic aspects of opioid withdrawal syndrome (Stella et al., 2003).

4.3.10 Cytokines
Evidence has affirmed the presence of a role of glial inflammatory response in the pathogenesis of opioid dependence. Astrocytes and microglia have been shown to respond to protracted opioid treatment in a pro-inflammatory manner by causing an upregulation of inflammatory cytokines (Raghavendra et al., 2002). Drugs that suppress glial inflammatory responses have been shown to suppress the expression of morphine withdrawal (Hutchinson et al., 2007). For example, AV411, a glial inhibitor that is noted to suppress glial production of inflammatory cytokines and chemokines and significantly decrease spontaneous opioid withdrawal (Hutchinson et al., 2007). Opioid-induced glial activation is reported to occur via non-classical opioid mechanisms, engaging the innate immune receptor, Toll-like-receptor 4 (TLR4), expressed on the glial cells (Hutchinson et al., 2007). Among the group of cytokines that are released following such an opioid-induced TLR4 response, interleukin-1β (IL-1β) plays a prominent role in mediating opioid dependence in laboratory animals (Hutchinson et al., 2007). A recent human genetic study that revealed an association between polymorphisms that alter IL-1β expression and risk of opioid dependence (Liu et al., 2009), providing the first clinical evidence supporting the pro-inflammatory opioid dependence hypothesis based on the involvement of cytokines. Following the production of IL-1β, other pro-inflammatory cytokines such as IL-6, and tumor necrosis factor-α (TNF-α) are induced which are in turn known to mediate chronic opioid effects (Raghavendra et al., 2002). Further, a recent study by Hao et al., (2011) has shown that chronic opioid exposure induces astrocytic activation to release TNFα in the PAG which in turn up-regulates the expression of pERK1/2, Fos, and pCREB subsequently causing the precipitation of a pronounced opioid withdrawal syndrome. However, data has also shown that morphine withdrawal results in a significant decrease in LPS-induced IL-12 synthesis in an in vivo model of morphine-withdrawal syndrome (Kelschenbach et al., 2005).
4.3.11 Orexin

Orexins (orexins A and B), also known as hypocretins (hypocretins 1 and 2), are multifunctional neuropeptides derived from a common precursor, preproorexin, which are transcribed in a discrete population of neurons in numerous brain regions, including the cortex, thalamus, hypothalamus, brain stem, and spinal cord (Matsuki and Sakurai, 2008). Orexin system is known to regulate multiple complex physiological functions like vigilance and sleep/wake cycle, feeding, appetite, reward seeking, and energy homeostasis (Kukkonen et al., 2002; Matsuki and Sakurai, 2008). Orexins orchestrate their diverse central and peripheral effects via two membrane-bound G protein-coupled receptors, OX1R and OX2R (Sakurai et al., 1998). Studies of acute opioid treatment have shown an attenuated increase in extracellular dopamine levels in the NAc by orexin receptor blockade in the ventral tegmental area (Narita et al., 2006). Moreover, behavioural studies have shown that the interaction between orexins and their receptors underlie the development of morphine-withdrawal related behavioural alterations (Georgescu et al., 2003). Further, morphine withdrawal has been shown to produce acute activation of orexin release as well as genetic expression from orexin neurons by a CRE activity and c-Fos linked pathway (Georgescu et al., 2003).

4.3.12 Corticotrophin releasing factor

Corticotropin-releasing factor (CRF) is a hypothalamic-releasing factor (Vale et al., 1981) that stimulates the release of adrenocorticotropin from the pituitary, which releases
glucocorticoids from adrenal glands. In addition, CRF is widely distributed throughout the brain and plays a major role in coordinating behavioral and autonomic responses to stress (Owens and Nemeroff, 1991). Alterations in the CRF system have been implicated in psychiatric illnesses that are precipitated by stress, such as depression and anxiety (Zorrilla and Koob, 2004). CRF is reported to mediate the anxiogenesis and aversive symptoms of opioid withdrawal (Koob, 2008). Two G protein-coupled receptors have been identified that bind CRF with high affinity: CRF receptor 1 (CRF1R) and CRF receptor 2 (CRF2R). CRF1R, which is involved in anxiety-related behavior (Bale and Vale, 2004) and reported to be expressed throughout the central nervous system has been shown to mediate several negative affective-like behavioural signs of morphine withdrawal (Iredale et al., 2000; Lu et al., 2000a; Contarino and Papaleo, 1999). Moreover, genetic disruption of the CRF2R pathway has shown to reduce the expression of major somatic signs of opiate withdrawal (Papaleo et al., 2008). The suppressive effect of CRF modulation on opioid withdrawal syndrome has been ascribed to its effect on the noradrenergic activity in the morphine withdrawn brain (Funada et al., 2001).

4.3.13 Fos

The transcription factor c-fos is commonly used as a marker of neuronal activity. Several studies have shown that opiate withdrawal increases the expression of the c-fos protein (Fos) both in brain regions associated with the physical (Hayward et al., 1990; Stornetta et al., 1993; Beckmann et al., 1995; Chieng et al., 1995; Rohde et al., 1996) and motivational aspects of opioid dependence (Stornetta et al., 1993; Rasmussen et al., 1995).
4.4 Novel pharmacological approaches for opioid withdrawal syndrome

4.4.1 Src Kinases

Src protein tyrosine kinase family is categorized into non-receptor tyrosine kinases and consists of nine members (Thomas and Brugge, 1997). Src, Fyn, Yes, and Yrk are ubiquitously expressed members of the family, whereas Blk, Fgr, Hck, Lck, and Lyn are expressed in more restricted patterns (Thomas and Brugge, 1997). Many of these src family kinases (cytoplasmic protein tyrosine kinases) are abundantly expressed in the central nervous system (Schlessinger, 2000). Acute opioid receptor activation is known to inhibit adenylyl cyclase (AC) activity (Law et al., 2000) but a sustained opioid receptor activation has been shown to cause a paradoxical super-activation of adenylyl cyclase mediated increase in the intracellular levels of cAMP. As described above this increased intracellular cAMP is the principal factor that causes the precipitation of withdrawal syndrome (Sharma et al., 1977; Law et al., 1982; Koob and Bloom, 1988; Pineyro and Archer-Lahlou, 2007). This observed change from opioid receptor-mediated AC inhibition to activation represents receptor signal switching. Chronic activation of opioid receptors has been shown to recruit the activated Src to form a complex with the opioid receptors and the $G_{a_{i2}}$ protein. The opioid receptor-$G_{a_{i2}}$-Src signaling complex causes the phosphorylation of the tyrosine residues present on Src-kinase and thus activates it, further enhancing the conversion of opioid receptor into the receptor tyrosine kinase like signaling complex. This opioid receptor-$G_{a_{i2}}$-Src-kinase complex, either directly or indirectly by activating other protein kinases, phosphorylates AC isoforms, such as AC5/6 and other signaling molecules, and eventually leads to the observed AC activation thus causing the observed ‘switching’ of the signals (Avidor-Reiss et al., 1996; Avidor-Reiss et al., 1997; Chakrabarti et al., 2001; Zhang et al.,
Continuous morphine treatment has been shown to cause the transcriptional increase in the intracellular levels of cSrc and Lyn, thus suggesting their potential role in opioid dependence (Zhang et al., 2009). Recently, src family kinases have been demonstrated to mediate continued opioid receptor activation based up-regulation of N-Methyl-D-Aspartic Acid (NMDA) receptors, which are in turn involved in the development of withdrawal syndrome in subjects having physiological dependence related to opioid use (Garzón et al., 2008; Sánchez-Blázquez et al., 2009). Further, it has been shown that Src mediate the activation of extra-cellular receptor kinases during continuous morphine treatment (Liu et al., 2011). Moreover, c-Src has been documented to directly phosphorylate G protein receptor kinase-2 (GRK2) and β arrestin2, which in turn direct the mechanistic changes in the functioning of G proteins caused by sustained opioid receptor activation (Sarnago et al., 1999; Walwyn et al., 2007; Hong et al., 2009). Additionally, a report has shown that sustained opioid receptor activation leads to a significant increase in the transcription of c-src tyrosine kinase (Loguinov et al., 2001). Further, pharmacological modulation of src family kinases has been demonstrated to alter the effects of sub-acute morphine administration in rodents (Narita et al., 2006; Garzón et al., 2008; Sánchez-Blázquez et al., 2009). Thus, it is suggested that the activation of src-family-kinases might play an important role in mediating the progression of opioid dependence and that the pharmacological manipulation of these enzymes exert an ameliorative effect on opioid withdrawal induced abstinence syndrome.

4.4.2 Nuclear factor-kappa-B

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB is found in almost all animal cell types and is
involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection (kappa light chains are critical components of immunoglobulins). Incorrect regulation of NF-κB has been linked to cancer, inflammatory & autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κB has also been implicated in processes of synaptic plasticity and memory. All proteins of the NF-κB family share a homology domain in their N-terminus. A subfamily of NF-κB proteins, including RelA, RelB, and c-Rel, has a transactivation domain in their C-termini. In contrast, the NF-κB1 and NF-κB2 proteins are synthesized as large precursors, p105, and p100, which undergo processing to generate the mature NF-κB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective degradation of their C-terminal region. Whereas the generation of p52 from p100 is a tightly-regulated process, p50 is produced from constitutive processing of p105. NF-κB dimers exist in a latent form in the cytoplasm bound by the IκB inhibitory proteins. NF-κB-inducing stimuli activate the IκB kinase complex (Karin et al., 2004) that phosphorylates IκB, leading to its ubiquitination and subsequent degradation in the canonical NF-κB activation pathway. IκB degradation exposes the DNA-binding domain and nuclear localization sequence of NF-κB and permits its stable translocation to the nucleus and the regulation of target genes. A prominent role for NF-κB transcription factors has been demonstrated throughout the immune system, where NF-κB-regulated gene expression is essential for processes of inflammation and host defense (Li and Verma, 2002). NF-κB transcription factors are expressed throughout the central nervous system in both in neurons and in non-neuronal cells, such as glia and Schwann cells. Further, NF-κB is a transcription factor which causes the activation of multiple downstream signals to the nucleus, resulting in
the regulation of a number of NF-κB-dependent genes responsible for the transcription of various cytokines, which are in turn implicated in mediating various effects of opioid drugs on the central nervous system (Chen et al., 2006). NF-κB is a crucial regulator of many physiological and patho-physiological processes based on neuronal excitability (Baeuerle, 1991). NF-κB and related factors have been reported to be transcribed in the brain cells (O’Neill and Kaltschmidt, 1997). Literature has shown that sub-chronic morphine treatment increases the activation state of the NF-κB signaling pathway. Further, NF-κB is also suggested to facilitate the development of rewarding aspects of chronic opioid treatment in rats (Zhang et al., 2011). NF-κB is a downstream molecule that is known to transmit the opioid receptor-mediated upstream signals to the nucleus, resulting in the regulation of the NF-κB-dependent genes, which are critical for the opioid functions and further enhancement of opioid receptor gene expression (Chen et al., 2006). Moreover, a report has shown that pharmacological modulation of NF-κB attenuates opioid withdrawal contraction in an in vitro model of opioid dependence (Capasso et al., 2001). Further, in our earlier study it has been demonstrated that diethyl dithiocarbamic acid, a relatively selective NF-κB inhibitor, exerts a beneficial effect on opioid withdrawal syndrome in a mouse model (Rehni et al., 2008a). Thus, nuclear factor kappa B activation has been proposed to be an important target mediating the progression of opioid withdrawal syndrome. Recently, NF-κB activation has been reported to control the transcription and biochemical activation of chemokines and thus regulate inflammatory processes, which are, in turn, proposed to precipitate withdrawal syndrome (Palma-Nicolas et al., 2010). Prolonged opioid treatment has been shown to enhance the transcription of chemokines and their respective receptors in the brain cells (Avdoshina et al., 2010). Moreover, opioid receptor activation linked chemokine stimulation has been implicated in mediating hypernociception associated
with chronic opioid use (White and Wilson, 2010). Chemokine C-C motif ligand 2 (CCL2) is a potent chemotactic cytokine protein that is released from morphine treated neurons in brain (Rock et al., 2006). Moreover, CCR2 activation has been implicated in the development of opioid abuse related human immunodeficiency virus-1 neuropathogenesis (El-Hage et al., 2006). However, the effect of pharmacological modulation of NF-κB activation linked chemokine activation on opioid withdrawal syndrome has not been examined.

4.4.3 Histone deacetylase

Transcription in cells is influenced by the manner in which DNA is packaged (Wade, 2001). In resting cells, DNA is tightly compacted to prevent accessibility of transcription factors. DNA is packaged into chromatin, a highly organized and dynamic protein-DNA complex. The fundamental subunit of chromatin, the nucleosome, is composed of an octamer of four core histones, i.e. an H3/H4 tetramer and two H2A/H2B dimers, surrounded by 146 bp of DNA (Ito et al., 2000; Strahl and Allis, 2000). Local chromatin architecture is an important factor in the regulation of gene expression. During activation of gene transcription, this compact, inaccessible DNA is made available to DNA binding proteins via modification of the nucleosome (Ito et al., 2000). This architecture of chromatin is strongly influenced by posttranslational modifications of the histones. Histone acetylation, a phenomenon known to play a critical role in facilitating transcription by modifying histone protein, occurs at the δ amino groups of lysine residues located at the N-termini. All core histones are acetylated in vivo (Wade, 2001). Steady-state levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases and histone deacetylases (HDACs) (Wade, 2001). In general, increased levels of histone acetylation (hyperacetylation)
are associated with increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) are associated with repression of gene expression (Ito et al., 2000; Forsberg and Bresnick, 2001; Wade, 2001). The fact that acetylation is a key component in the regulation of gene expression has stimulated the study of histone deacetylases (HDACs) in relation to the aberrant gene expression often observed in cancer (Cress and Seto, 2000). Histone deacetylases (HDAC) are ubiquitous enzymes that exist in 18 isoforms which are categorized into various groups. Class I includes HDAC1, HDAC2, HDAC3 and HDAC8; Class IIA includes HDAC4, HDAC5, HDAC7 and HDAC9 and; Class IIB includes HDAC6 and HDAC10; Class III HDACs include sirtuins which are relatively less sensitive to the existing broad spectrum pharmacological modulators of histone deacetylases and; Class IV includes HDAC11 (Dokmanovic et al., 2007) (Figure 2).

Recent evidence has shown that histone deacetylase positively modulates the activity of cAMP response element binding protein (CREB) (Shen et al., 2008). In turn, CREB participates in the feed-forward loop leading to the characteristic AC super-activation linked with opioid withdrawal syndrome. Moreover, studies have implicated the involvement of histone deacetylase in mediating cAMP-CREB-AC8 pathway dependent pathway leading to super activation of adenylyl cyclase and resultant up-regulation of the cAMP pathway, a biochemical phenomenon known to mediate opioid withdrawal syndrome (Liu and Anand, 2001; Nestler, 2004). Further, opioid receptor regulation, an important molecular event leading to the development of opioid dependence, has been reportedly ascribed to histone deacetylase mediated epigenetic control of transcription promoters (Hu et al., 2001; Hwang et al., 2007). Additionally, pharmacological inhibition of histone deacetylase has been shown to enhance the transcription of opioid receptors in neurons (Hu et al., 2001; Hwang et al., 2007). Moreover, previous studies have shown that histone deacetylase inhibition results in the
reversal of a number of measures of behavioural plasticity linked with opioid addiction viz., behavioral sensitization associated with single morphine exposure (Jing et al., 2011), morphine-induced locomotor sensitization and conditioned place preference (Sanchis-Segura et al., 2009; Wang et al., 2010). Thus, it is suggested that the activation of histone deacetylase might play an important role in mediating the progression of opioid dependence and that the pharmacological manipulation of this enzyme might exert an ameliorative effect on opioid withdrawal induced abstinence syndrome. Histone deacetylase inhibition is noted to modulate the activity of CREB by an interleukin 1- β converting enzyme (IL-1β) associated pathway (Suzuki et al., 2003). Further, it has been observed that an elevated level of hippocampal expression of IL-1β is associated with opioid withdrawal induced jumping behavior in mice (Liu et al., 2011). Thus, interleukin-1β converting enzyme over-activation might be involved in the potential histone deacetylase linked precipitation of opioid dependence related withdrawal syndrome.
Figure 2: Various aspects of the transcription process and its regulation by histone acetylation (HAT= Histone Acetyltransferase; HDAC=Histone Deacetylase; H=Histone).
4.4.4 Tyrosine Phosphatase

A cornerstone of many cell-signalling events rests on the reversible phosphorylation of tyrosine residues on proteins. The reversibility relies on the co-ordinated actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs), both of which exist as large protein families (Stoker, 2005). Besides scavenging phosphotyrosine, the PTPs are noted to specifically regulate a wide range of signalling pathways. Chronic activation of opioid receptors has been shown to form an opioid receptor-\(\text{G}_\alpha_{i2}\)-c-SRC-Src-kinase complex which leads to adenylyl cyclase (AC) activation via a tyrosine phosphorylation associated mechanism (Avidor-Reiss et al., 1996; Avidor-Reiss et al., 1997; Chakrabarti et al., 2001; Zhang et al., 2009). It has been reported by O’Connor et al (1995) that tyrosine phosphatase induced dephosphorylation is a prerequisite for the activation of opioid receptor-\(\text{G}_\alpha_{i2}\)-c-SRC-Src-kinase complex dependent AC super-activation based development of physiological dependence associated precipitation of opioid withdrawal syndrome. Moreover, Ishiguro et al. (2008) have revealed that a polymorphism of gene responsible for transcription of protein tyrosine phosphatase receptor type beta (PTPRB) is associated with substance abuse vulnerability in man. Further, chronic morphine treatment has been shown to up-regulate PTPRB expression in locus coeruleus neurons (Ishiguro et al., 2008; Paul and Lombroso, 2003). Further, chronic activation of opioid receptor linked superactivation of cAMP pathway or CREB in the locus coeruleus neurons has been shown to cause an upregulation of tyrosine phosphatase (McClung et al., 2005). Thus, it is suggested that the activation of tyrosine phosphatase might be contributing towards the development of opioid dependence.
4.4.5 Calpain

The calpain system is calcium activated neutral protease system that leads to an irreversible proteolytic processing of target proteins. It comprises of three molecules viz., μ-calpain, m-calpain and calpastatin (Goll et al., 2003). Calpains are a set of important calcium sensitive enzymes that are reported to mediate various calcium dependent molecular events which modulate synaptic plasticity in brain cells, a phenomenon contributing to the development of physiological dependence associated precipitation of opioid withdrawal syndrome (Drdla et al., 2009; Zadran et al., 2010). Literature has shown that calpain, a calcium activated neutral cysteine endopeptidase, catalyses the proteolytic activation of Gaα proteins (Sato-Kusubata et al., 2000) which in turn mediates adenylyl cyclase super-activation linked causation of opioid withdrawal syndrome (Trujillo and Akil, 1991; Trujillo and Akil, 1995; Zang et al., 2000; Sweitzer et al., 2004a; Esmaeili-Mahani et al., 2008; Drdla et al., 2009; Zadran et al., 2010). Therefore, calpains may mediate the biochemical progression of opioid dependence based withdrawal syndrome.

4.5 Pharmacological Interventions Employed in the Present Study

**SU-6656:**
A study of Blake et al. (2000) has shown that SU6656 is a selective inhibitor for the Src family of tyrosine kinases.

**Ammonium pyrrolidine dithiocarbamate:**
Ammonium pyrrolidine dithiocarbamate (APD) is a selective inhibitor of NF-κB (Schreck et al., 1992).
RS 102895:
RS 102895 is a selective CCR-2 chemokine receptor antagonist (Mirzadegan et al., 2000; Onuffer et al., 2002).

Tributyrin:
Tributyrin is a selective histone deacetylase inhibitor (Chen and Breitman, 1994; Rocchi et al., 2005).

Trichostatin A:
Trichostatin A is a selective histone deacetylase inhibitor (Yoshida et al., 1995).

N-Acetyl-Asp-Glu-Val-Asp-al:
N-Acetyl-Asp-Glu-Val-Asp-al is a selective interleukin-1β converting enzyme inhibitor (Margonin, 1997).

Sodium orthovanadate:
Sodium orthovanadate is a selective inhibitor of tyrosine phosphatase (McLauchlan et al., 2010; Sugano et al., 2004).

SJA 7019:
SJA 7019 is a selective inhibitor of calpain (Liu et al., 2002).