INTRODUCTION

International Association for the study of Pain (IASP) defined neuropathic pain as “Pain initiated or caused by a primary lesion or dysfunction in the nervous system” (Burket et al., 2003). The development of peripheral, as well as central sensitization, is the key feature during the damage of the nervous system and sensitization of the nerves occurs due to the loss of inhibitory controls (Attal et al., 2006; Gwak et al., 2006). The symptoms due to nerve damage may include numbness, tingling, spontaneous pain, hyperalgesia, allodynia, dysesthesia and other sensory abnormalities (Jensen et al., 2011; Khangura et al., 2017). Depending on the location of the nerve damage, types of nerve affected and etiology, there are different types of neuropathies including peripheral neuropathy, cranial neuropathy, autonomic neuropathy, diabetic neuropathy, drug-induced neuropathy, and alcoholic neuropathy, etc. (Haga et al., 2015; Zeng et al., 2017). It is estimated that more than 30% of the general population is affected by persisting pain, which often becomes pathological and debilitating, and causes people to seek medical attention (Ji et al., 2014; Li et al., 2014) and around 7 in every 100 people over the world have chronic neuropathic pain (Colloca et al., 2017).

The therapeutic approaches of neuropathic pain include different classes of drugs including anti-convulsant (gabapentin, pregabalin, carbamazepine, and lamotrigine) (Backonja, 2002), anti-depressants (amitriptyline, nortriptyline, lofepramine duloxetine and venlafaxine) (Kim, Abdi, 2014), opioids (morphine, oxycodone, propoxyphene) (Navratilova et al., 2015), N-methyl-D-aspartate (NMDA) receptor antagonists (ketamine, methadone, amantadine, dextromethorphan,
and memantine) (Gagnon et al., 2003; Carlsson et al., 2004) and topical agents (tramadol, codeine, and dihydrocodeine) (Jaggi, Singh, 2011; Stanos, Galluzzi, 2013). Various herbal preparations containing phytomedicines such as curcumin, sodium ferulate, safranal, tanshinone IIA, geniposidic Acid, limonoids, and vitamin E are also shown to ameliorate neurodegeneration and neuropathic pain (Tamaddonfard et al., 2014; Cao et al., 2015; Meng et al., 2015; Venkatesan et al., 2015; Chu et al., 2016).

Nevertheless, gabapentin is clinically proven, effective treatment for the management of neuropathic pain (Dworkin et al., 2007; Jang et al., 2018). Federal Drug Administration (FDA) and CDC guidelines also recommend gabapentinoids as the first-line drug for the treatment of neuropathic pain (Luo et al., 2017). Gabapentinoids include gabapentin and pregabalin and these have shown promising results in different types of neuropathies including diabetic neuropathy (Ramsay, 1994), postherpetic neuralgia (Backonja et al., 1998), migraine (Rowbotham et al., 1998) and pain associated with cancer and multiple sclerosis (Di Trapani et al., 2000). Studies have shown that gabapentin inhibits ectopic discharge from the injured peripheral nerves and attenuates spontaneous pain (Abdi, Lee, Chung, 1998; Pan et al., 1999; Luo et al., 2017). The broad spectrum of pharmacological activity of gabapentin has led several investigators to determine its mechanism of action. The neuropathic pain attenuating effects of gabapentin have been attributed to their actions on the central nervous system (at the spinal cord or the brain) due to enhanced inhibitory input on the GABA-mediated pathways, which leads to a reduction in the excitatory action potential (Cai et al., 2012). Furthermore, antagonism of NMDA receptors and blockade of calcium channels in the central system CNS or inhibition at the peripheral nervous system may also contribute in attenuating pain (Abdi, Lee, Chung, 1998; Dooley et al., 2000). Gabapentin has also been shown to modulate other targets including transient receptor potential channels, protein kinase C and inflammatory cytokines (Vellani, Giacomoni, 2017). It may also act on the supra-spinal region to stimulate noradrenaline-mediated descending inhibition, which contributes to attenuating neuropathic pain (Kukkar et al., 2013). However, its precise mechanisms are not clearly defined yet (Billie et al., 2006).

Milrinone is a biguanide compound and documented as a selective phosphodiesterase-3 inhibitor. It significantly inhibits cAMP phosphodiesterase activity, increasing intracellular cAMP levels (Alousi, Johnson, 1986). Owing to its action (selective increase in cAMP levels), it has been used in clinics as an inodilator agent in the management of congestive heart failure (CHF). However, its prolonged use is not beneficial in CHF patients; rather it increases the mortality rate in these patients. Accordingly, it is employed for short term management of CHF during the decompensation state (Hilleman, Forbes, 1989; Landmesser, Drexler, 2007).

Adenosine 3’, 5’cyclic monophosphate (cAMP) is a key second messenger in various signal transduction pathways and it regulates numerous cellular functions, including cell growth and differentiation, gene transcription and protein expression (Yan et al., 2016). Studies have shown the key participation of cAMP in neuropathic pain and it has been shown that an increase in cAMP levels may be important in the development of neuropathic pain (Bie et al., 2005; Hugo, Levine, 2007). Moreover, drugs/interventions that decrease the levels of intracellular cAMP are shown to attenuate pain in different experimental models (Shao et al., 2016). There exists a relationship between cAMP and Ca^{2+} channel as an increased level of cAMP increase protein kinase A activity, which sequentially promotes the opening of the L-type calcium channel resulting in calcium entry into the cell (Earl, Linden, Weglicki, 1986). Many studies have implicated the cAMP-calcium-protein kinase A pathway in neuropathic pain conditions (Guindon et al., 2008).

It has been shown that gabapentin reduces intracellular calcium current in injured as well as in the control of primary afferent neurons (Sarantopoulos et al., 2002). Other studies have also shown that gabapentin decrease intracellular calcium levels (Martin et al., 2002). Gabapentin has been shown to attenuate the expression of phosphorylated cAMP response elements in the amygdala (Li et al., 2010) suggesting the possible role of cAMP in gabapentin mediated analgesic actions. However, there is no study reporting the influence of...
cAMP modulating drugs on the neuropathic pain attenuating the actions of gabapentin. Owing to PDE-3 inhibitory activity, milrinone increases the cAMP levels. Accordingly, milrinone was employed as a pharmacological tool to investigate the role of cAMP in neuropathic pain attenuating actions of gabapentin in chronic constriction injury model in rats. In other words, to explore whether the decrease in cAMP levels plays an important role in gabapentin-mediated pain attenuating action, a pharmacological agent which increases the intracellular cAMP levels (milrinone) was employed in this study. The attenuation of beneficial effects of gabapentin in the presence of an agent that increases the cAMP levels (milrinone) may confer the importance of cAMP in gabapentin-mediated pain attenuating actions. Therefore, the present study was designed to investigate the possible role of cAMP in pain attenuating actions of gabapentin by employing milrinone as a pharmacological tool (as a cAMP elevating agent) in chronic constriction injury in rats.

MATERIAL AND METHODS

Experimental Animals

All experiments were performed as per approval by the Institutional Animal Ethics Committee (IAEC) (Reg. no.1407/PO/Re/S/11CPCSEA). Sprague Dawley rats of either sex, weighing 200–250 g were purchased from the Institute of Microbial Technology, Chandigarh. They were housed in the departmental animal house with standard laboratory conditions i.e. temperature 23±2°C (Baumans, Van Loo, 2013), chow diet and the normal cycle of 12 hours light and 12 hours dark. The care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Ministry of Environment and Forest, Government of India. (Approval Number: ATRC/04/18).

Drugs and Reagents

Gabapentin (Gabantin™, 500 mg) was purchased from Sun Pharmaceuticals Industries, India. Milrinone was obtained from Neon Laboratories limited, Mumbai. All the chemicals used in the present study were of analytical grade.

Induction of Neuropathy Pain by Chronic Constriction Injury (CCI)

Neuropathic pain was induced in rats by chronic constriction injury (Bennett, Xie, 1988). Rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg i.p.) (Wang et al., 2017). With proper surgical care, the hair of the rat’s lower back and thigh region was shaved and the skin was sterilized with a 0.5% povidone solution. The left thigh was cut and a cut made directly through the biceps femoris muscle to expose the sciatic nerve. After exposure, the sciatic nerve was ligated with a silk 4-0 thread at four sites with a 1 mm gap. The care was taken to tie the ligatures around the nerve so that the nerve was barely constricted. The ligation affected approximately 6 mm of the nerve length (Sumizono et al., 2018). The muscle and skin were closed in two layers with the use of thread and topical antibiotic was applied. All surgical procedures were carried out under normal sterile conditions.

Behavioral Examination

Cold allodynia (acetone test)

The cold allodynia was assessed by spraying a 100 μL of acetone onto the surface of the paw of rat (placed over a wire mesh), without touching the skin. The response of rat to acetone was noted for the 20s and was graded to a 4-point scale as defined by Flatters and Bennett (Flatters, Bennett, 2004). 0: no reflex; 1: quick stamp, flick or withdrawal of the paw; 2: repeated flicking or prolonged withdrawal; and 3: repeated flicking with the licking of the paw. Acetone was applied three times to the hind paw, with a gap of 5 min between the acetone applications and the individual scores noted in a 20s interval were added to obtain a single score over a cumulative period of 60s. The minimum score was 0, while the maximum possible score was 9 (Kukkar et al., 2013).
**Heat hyperalgesia (hot-plate test)**

The heat hyperalgesia was assessed by Eddy’s hot-plate, as an index of thermal hyperalgesia, by measuring the thermal nociceptive threshold. The animals were placed on the hot-plate at a temperature of 52.5 ± 1.0 °C and withdrawal latency, in terms of the licking of the hind paw, was recorded in seconds. The maintained cut-off time was 15 sec (Jain et al., 2009).

**Mechanical hyperalgesia (pinprick test)**

Mechanical hyperalgesia was measured by the pinprick test (Erichsen, Blackburn-Munro, 2002). The injured surface of the hind paw was touched with the point of a bent gauge needle (at 90° to the syringe) at the strength necessary to produce a reflex withdrawal response. The paw withdrawal duration (PWD) was recorded in seconds and the normal quick reflex withdrawal response was given the value of 0.6 s.

**Experimental Protocol**

Ten groups, each comprising five rats, were employed in the present study.

**Group I: Normal Control**

In the normal control group, rats were not subjected to any treatment. The different behavioral tests, including the heat hyperalgesia, cold allodynia, and mechanical hyperalgesia were employed on day 0 (a day before surgery) and 14th day (post-surgery).

**Group II: Sham Control**

In the sham control group, rats were subjected to the surgical procedure to expose the left sciatic nerve on day 1 without any nerve ligation. The behavioral tests including the heat hyperalgesia, cold allodynia, and mechanical hyperalgesia were conducted before doing surgery on day 0 (a day before surgery) and 14th day (post-surgery).

**Group III: CCI Control**

In this group, rats were subjected to the surgical procedure to expose and ligate the left sciatic nerve on day 1. The pain-related behavioral tests were performed at different time intervals as described in group I.

**Group IV: Gabapentin (100 mg/kg) in CCI**

In this group, gabapentin (100 mg/kg) was administered in CCI subjected rats for 14 days, starting from day 1 (day of surgery). The pain-related behavioral tests were performed at different time intervals as described in group I.

**Groups V, VI and VII: Milrinone (10, 30 and 50 mg/kg i.p.) in Gabapentin + CCI**

Milrinone (10, 30 and 50 mg/kg) was injected 30 min before gabapentin administration for 14 days in CCI-subjected rats, starting from day 1. The pain-related behavioral tests were performed at different time intervals as described in group I.

**Group VIII: Milrinone (50 mg/kg) in CCI**

Milrinone (50 mg/kg) was administered in CCI subjected rats for 14 days, starting from day 1 (post-surgery). The pain-related behavioral tests were performed at different time intervals as described in group I.

**Group IX: Milrinone (50 mg/kg) per se**

Milrinone was administered in normal rats for 14 days. The pain-related behavioral tests were performed at different time intervals as described in group I.

**Group X: Gabapentin (100 mg/kg) per se**

Gabapentin was administered in normal rats for 14 days. The pain-related behavioral tests were performed at different time intervals as described in group I.
**Statistical Analysis**

The results were expressed in mean ± S.D. The data of behavioral tests were analyzed using two-way ANOVA followed by Bonferroni’s *post hoc* test, using Graph pad prism version-5.0 software. The *P*-value < 0.05 was considered to be statistically significant.

**RESULTS**

**Effect of Pharmacological Interventions on Cold-Alloodynia (Acetone Drop Test) in Chronic Constriction Injury-induced neuropathic pain**

Chronic constriction injury resulted in significant development of cold alldynia on 14th-day post-surgery (Figure 1) as compared to the sham group, measured by acetone drop test. Administration of gabapentin (100 mg/kg i.p.) for 14 days significantly attenuated CCI-induced cold alldynia in comparison to the sham group. There was a significant decrease in alldynia scores in gabapentin treated CCI rats. Pretreatment with milrinone (30 and 50 mg/kg, i.p.), for 14 days, attenuated gabapentin-induced decrease in alldynia score in CCI-subjected rats. However, milrinone (10 mg/kg) did not show a significant effect on the alldynia gabapentin treated CCI rats. Pretreatment with milrinone (50 mg/kg i.p.) led to a significant increase in cold alldynia score in CCI-subjected rats. *Per se* administration of milrinone (50 mg/kg i.p.) and gabapentin did not modulate cold alldynia in normal rats.

**FIGURE 1** - Effect of pharmacological interventions on chronic constriction injury-induced paw cold alldynia assessed by acetone drop test. Values are given in mean ± S.D., n=5 rats per group; Two-way ANOVA followed by Bonferroni’s *post hoc* test. *a* *P* < 0.05 vs sham control, *b* *P* < 0.05 vs chronic constriction injury, *c* *P* < 0.05 vs gabapentin in CCI.

**Effect of pharmacological interventions on heat-hyperalgesia (hot plate test) in chronic constriction injury-induced neuropathic pain**

Chronic constriction injury significantly decreased paw withdrawal latency in a hot plate test as compared to the sham group (Figure 2), signifying the development of heat-hyperalgesia. Administration of gabapentin (100 mg/kg i.p.), for 14 days, attenuated chronic constriction injury-induced development of heat-hyperalgesia in a significant manner. Pretreatment with milrinone (30 mg/kg and 50 mg/kg i.p.) for 14 days abolished gabapentin-
induced decrease in paw withdrawal latency in CCI-subjected rats. However, milrinone (10 mg/kg) did not modulate the effects of gabapentin in CCI-subjected rats. Administration of milrinone (50 mg/kg i.p.) for fourteen days significantly decreased paw withdrawal latency in CCI-subjected rats. Per se administration of milrinone (50 mg/kg i.p.) and gabapentin did not modulate heat-related behavioral functions in normal rats.

Effect of pharmacological intervention on mechanical hyperalgesia (pinprick test) in chronic constriction injury-induced neuropathic pain

Chronic constriction injury led to a significant increase in paw withdrawal duration in response to pinprick test, as compared to the sham group (Figure 3), suggesting the development of mechanical hyperalgesia. Treatment with gabapentin (100 mg/kg i.p.), for 14 days, attenuated CCI-induced increase in withdrawal duration in a significant manner. Pretreatment with milrinone (30 mg/kg and 50 mg/kg, i.p.), for 14 days, abrogated the effects of gabapentin on paw withdrawal duration in CCI-subjected rats. Milrinone (10 mg/kg) did not modulate the behavior of gabapentin in CCI-subjected rats. Treatment with milrinone (50 mg/kg i.p.) for 14 days significantly increased paw withdrawal duration in CCI-subjected rats. Per se administration of milrinone (50 mg/kg i.p.) and gabapentin did not modulate mechanical pain-related behavioral functions in normal rats.
The goal of the present study was to elucidate the role of cAMP in gabapentin-mediated pain attenuating effects in chronic constriction injury model in rats. To accomplish this, the present study employed the chronic constriction injury model to induce neuropathic pain (Bennett, Xie, 1998; Jaggi et al., 2011; Ko et al., 2015). In the present study, CCI-subjected rats showed significant enhancement in paw withdrawal in response to acetone application on injured paw. The observed cold allodynic response in the CCI-subjected rats in the form of increased withdrawal duration mimics the symptoms of cold allodynia in patients suffering from complex regional pain syndrome (Kemler, de Vet, 2000; Tahmoush et al., 2000). Furthermore, there was also a significant development of mechanical and heat hyperalgesia, observed on the 14th day after surgery. The development of mechanical hyperalgesia was observed by noting an increase in paw withdrawal duration in response to pin prick test, while the development of heat hyperalgesia was observed by noting a decrease in paw withdrawal latency in hot plate test. These results observed in the present study are in line with previous findings (Bennett, Xie, 1988; Flatters, Bennett, 2004; Khangura et al., 2017). Earlier studies have documented that the pain-related behavioral alterations are at peak on the 14th day in chronic constriction subjected rats (Jaggi, Singh, 2011; Kukkar et al., 2014).

In the present study, administration of gabapentin for 14 days significantly attenuated CCI-induced pain-related behavioral alterations including paw cold allodynia, mechanical and heat hyperalgesia. Earlier studies have also observed the beneficial effects of gabapentin on the behaviors of both thermal and mechanical stimulations in CCI-induced rats (Yeh et al., 2011; Kukkar et al., 2013). As per the FDA and CDC guidelines, gabapentinoids (pregabalin or gabapentin) are the first-line drugs for the treatment of neuropathic pain (Luo et al., 2017).

Gabapentin is a well established anti-epileptic and neuropathic pain attenuating drug, however, its underlying molecular mechanisms are not entirely clear (Billie et al., 2006). It has been reported that gabapentin decreases spontaneous neuronal activity by binding to the α2δ-1 subunit of voltage-gated calcium channels (VGCC), and thereby reducing neuronal calcium currents (Gee et al., 1996; Shimoyama et al., 2000). Gabapentin has also been shown to decrease cAMP levels in the brain.
regions (Li et al., 2010). Considering the close association between cAMP and Ca\(^{2+}\) signaling (Hofer, 2012), it was hypothesized that there may be a key role of cAMP in gabapentin-mediated neuropathic pain attenuating actions. Therefore, to explore the role of cAMP in gabapentin-mediated pain attenuating effects, as a cAMP elevating agent, milrinone (as a pharmacological agent) was co-administered in gabapentin-treated CCI rats. In the present study, pretreatment with milrinone (30 mg/kg and 50 mg/kg) for 14 days significantly abolished neuropathic pain attenuating actions of gabapentin in CCI-subjected rats. Since milrinone administration leads to an increase in the cAMP levels, therefore, it is proposed that milrinone-induced increase in intracellular cAMP levels may contribute to abolishing the analgesic actions of gabapentin in the present study.

The cAMP is a key second messenger and it modulates numerous physiological as well pathophysiological functions in the body (Yan et al., 2016). Amongst different functions, studies have shown that an increase in the levels of cAMP increases the pain sensitization (Shao et al., 2016). It has been shown that an increase in pain sensitization during morphine withdrawal is due to the potentiation of a cAMP-linked signaling pathway (Bie et al., 2005). Many other studies have implicated that cAMP-protein kinase A pathway triggers hyperexcitability in sensory neurons of dorsal root ganglia (Guindon et al., 2008). Moreover, studies have also demonstrated that inhibition of cAMP attenuates neuropathic pain (Liou et al., 2007; Shao et al., 2016). The key role of cAMP in increasing pain sensitization was also supported by the results of the present study showing that treatment with milrinone (50 mg/kg) exacerbated pain intensity in CCI-subjected rats. However, milrinone did not modulate pain-related behavior parameters in normal rats suggesting that cAMP may selectively increase pain sensitization during nerve injury, without any significant alteration in non-injured conditions. Gabapentin has also been shown to decrease cAMP levels in the brain regions (Li et al., 2010). The results of the present study provide evidence that the analgesic actions of gabapentin are dependent on the intracellular cAMP levels and an increase in cAMP levels may attenuate the analgesic actions of gabapentin.

**CONCLUSION**

The attenuation of gabapentin-mediated analgesic actions in the presence of milrinone suggests that an increase in intracellular cAMP following milrinone administration significantly contributes to abolishing the analgesic actions of gabapentin in the CCI-subjected rats. In other words, a decrease in cAMP plays a key role in gabapentin-mediated pain attenuating actions in CCI-subjected rats.

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