CHAPTER 4: Loperamide, a peripheral Mu-Opioid receptor agonist, attenuates chemotherapy-induced neuropathic pain in rats

Chapter-4

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4.1 Introduction

Chemotherapy-induced neuropathic pain (CINP) is a noteworthy complication stemming from the administration of chemotherapeutic agents, affecting around 50-90% of individuals undergoing chemotherapy treatment [7]. CINP manifests as a painful condition which can progress in severe cases to loss of sensory perceptions. Impairments in sensory functions can lead to a lowered pain threshold in response to various stimuli giving rise to mechanical allodynia, tingling, burning, paraesthesia and dysesthesia triggered by contact with warm or cool temperatures [35,256-258]. Despite its increasing recognition, CINP continues to pose a significant challenge, as it currently lacks viable curative approaches [4]. Some of the medications currently being used for CINP include SNRIs such as duloxetine, anticonvulsants like gabapentin and pregabalin, TCAs and patches of lidocaine, capsaicin and both weak and strong opioids [7]. Unfortunately, a substantial proportion, approximately 70%, of patients does not get effective pain relief from these medications [259]. Moreover, most of these drugs primarily works by targeting the higher pain centers, present in the CNS and are associated with several unwanted side effects such as hepatic impairment, renal insufficiency, fatigue and central toxicities like anxiety, dizziness, sedation, respiratory depression, cognitive dysfunction, addiction, abuse potential [260].

Nonetheless, a growing body of clinical evidence suggests the involvement of the peripheral nervous system (PNS) in the progression and maintenance of chronic pain [261]. Targeting peripheral nociceptors modulate pain signals at their source, thereby relieving the pain with higher specificity, and minimizing off-target CNS side effects. Thus, targeting the PNS for development of safer therapeutics devoid of central side effects and toxicities is of paramount importance. Recent studies suggest that targeting MOR using peripherally restricted agonists alleviates heat and mechanical hypersensitivity without producing CNS toxicities [262-264].

Loperamide, a peripheral mu opioid receptor (MOR) agonist has previously been evaluated for the attenuation of inflammatory pain, neuropathic pain and bone cancer pain [215,265]. The limited CNS penetration of loperamide is due to the efflux by P-glycoprotein that prevents circulating loperamide from effectively crossing the blood–brain barrier [214]. The present study is designed with an aim to investigate the effectiveness of systemic loperamide as a potential therapeutic approach for the management of paclitaxel induced evoked and ongoing pain. Our investigations delved into novel pathophysiological mechanisms that contribute to the emergence of paclitaxel induced neuropathic pain, encompassing crosstalk between TRP channels, VGSCs, NR2B, and neuroinflammatory signaling. Using molecular biology tools, we investigated the mechanism of action of loperamide responsible for attenuation of chemotherapy-induced evoked and ongoing pain in rats.

4.2 Experimental procedure

The animals were divided into six groups: Naïve; Paclitaxel (PTX) + Vehicle (20% beta-cyclodextrin (CDEX) in 0.9% sterile saline); Paclitaxel + Loperamide (5, 10 and 20 mg/kg, *s.c.*); and Paclitaxel + Gabapentin (60 mg/kg, *s.c.*) . Paclitaxel was diluted using 0.9% normal saline to make a working concentration of 2mg/ml, from its commerically available concentration of 6mg/ml. Loperamide hydrochloride was dissolved in 20% CDEX in 0.9% normal saline solution and gabapentin was dissolved in 0.9% normal saline. The rats were acclimatized in the test room for about 2-3 days to get habituated with the enviornment, followed by which pre-PTX baseline testing was performed for all the behavioral parameters. After completing the baseline pain behavioral testing in rats, neuropathic pain was induced by administering repeated paclitaxel injections. Twenty eight days post first paclitaxel injection i.e., once the pain response peaked, animals were treated with different doses of loperamide , vehicle and gabapentin. The time of administration was considered as 0 min and the evoked pain behavioral assays were performed at 30, 60, 120 and 240 mins post-drug or vehicle administration. Afterwards, conditioned place preference assay was conducted for the assesment of spontaneous pain in rats followed by CNS toxicity testing, using open field and rota-rod tests. After completion of the behavioural studies, animals were euthanized on day 45 and their sciatic nerve, DRGs and lumbar spinal cord was harvested for carrying out the mechanistic studies.

4.3 Results and discussion

4.3.1 Activation of peripheral MOR attenuates pain-like behaviour in PTX-induced neuropathic rats

Paclitaxel administration resulted in development of mechanical allodynia and hyperalgesia in rats. The vehicle treated rats showed a significant decrease in the paw withdrawal threshold (PWT) to non-noxious mechanical stimuli in the hind paw as compared to their respective pre-paclitaxel baselines and naïve rats ($p < 0.001$). The three doses of loperamide (a peripheral MOR agonist) significantly improved the PWT in hind paw with effect starting from 30 mins, post drug administration. At 120 mins we observed the peak therapeutic effect in hind paw (5, 10, and 20 mg/kg s.c.; $p < 0.001$) as compared to their pre-drug baselines and vehicle treated PTX group. The effect lasted upto 240mins post drug administration (Figure. 4.1A). Gabapentin (60mg/kg s.c.) showed a significant effect from 30mins ($p < 0.05$), which lasted up to 240 mins post-drug administration ($p < 0.001$) as compared to the vehicle treated PTX rats.

Paclitaxel administration significantly increased the paw withdrawal frequency to noxious mechanical stimuli in both the paws as compared to the respective pre-paclitaxel baselines and naïve rats ($p < 0.001$). The three doses of loperamide significantly decreased the PWF in hind paw with effect starting from 30 mins, post drug administration. At 120 mins we observed the peak therapeutic effect in hind paw (5 mg/kg; $p < 0.05$ and 10 and 20 mg/kg; $p < 0.001$) as compared to their pre- drug baselines and vehicle treated PTX rats (Figure. 4.1B). Gabapentin (60mg/kg, s.c.) showed a significant effect from 30mins ($p < 0.05$), which peaked at 120mins ($p < 0.001$) and gradually declined by 240mins as compared to the vehicle treated PTX rats.

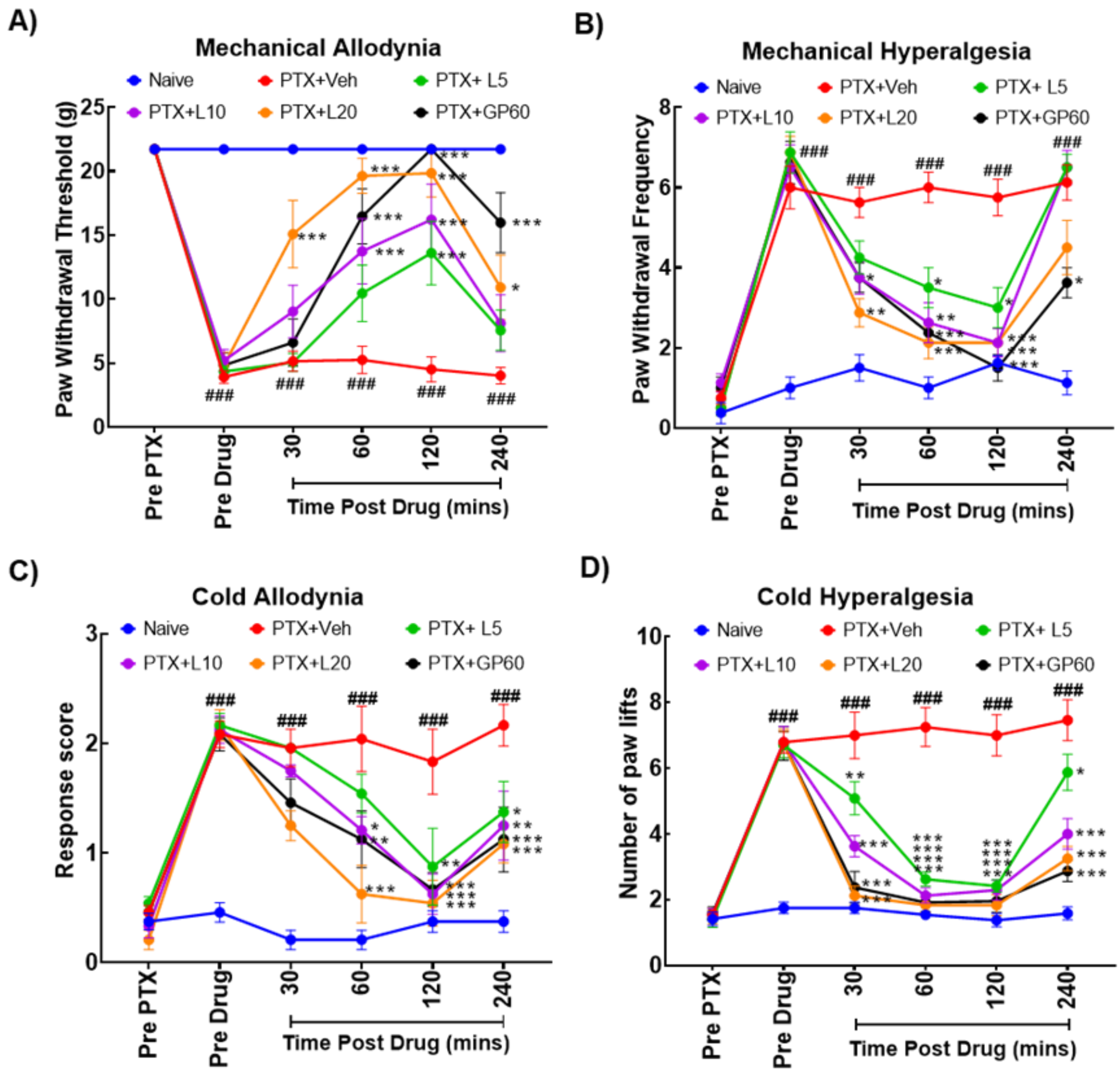

Figure. 4.1. Effect of peripheral MOR agonist, loperamide, on CINP-induced mechanical and cold hypersensitivity in rats. (A) von Frey hair test: Loperamide (5, 10, 20 mg/kg s.c.) and gabapentin (60 mg/kg s.c.) treatment significantly inhibits paclitaxel-induced hypersensitivity to non-noxious mechanical stimuli. **(B) Pin prick test:** Paclitaxel administration significantly increases the paw withdrawal frequency as compared to the pre-injury baseline in response to noxious mechanical stimuli. Loperamide (5, 10, 20 mg/kg s.c.) and gabapentin (60mg/kg s.c.) treatment significantly reduced the paw withdrawal frequency of PTX administered rats as compared to their pre-drug baseline. **(C) Acetone spray test:** Paclitaxel induces significant increase in paw withdrawal score of rats in response to non-noxious cold stimuli, which was alleviated upon treatment with loperamide (5, 10 & 20 mg/kg s.c.) and gabapentin (60mg/kg s.c.). **(D) Ice floor test:** Paclitaxel administration led to the development of cold hypersensitivity in rats, evident from the higher count of paw lifts compared to their pre-injury baseline. Administration of loperamide (5, 10 & 20 mg/kg

s.c.) and gabapentin (60 mg/kg s.c.) significantly decreased the number of paw lifts in response to noxious cold stimuli. Data was expressed as mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparison) (n=8). ### ($p<0.001$) represents significance compared to Naïve group. *($p<0.05$), **($p<0.01$), ***($p<0.001$) represents significance compared to PTX+ Vehicle group. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin (GP60): 60mg/kg.

Paclitaxel administration also resulted in development of significant cold allodynia and cold hyperalgesia in rats. Loperamide treatment significantly reversed cold hypersensitivity in both the behavioural assays in PTX-induced neuropathic rats. Paclitaxel significantly increased the response score to non-noxious cold stimuli in hind paw as compared to the respective pre-paclitaxel baselines and naïve rats ($p <0.001$) which was significantly attenuated by loperamide with effect starting from 60 mins, post drug administration. At 120 mins we observed the peak therapeutic effect (5mg/kg; $p<0.01$, 10 and 20 mg/kg; $p < 0.001$) as compared to their pre- drug baselines and vehicle treated rats (Figure. 4.1C). The effect of the drug in response to non-noxious cold stimuli, started at a later time point as compared to the mechanical stimuli but lasted upto 240 mins post drug administration. Gabapentin (60mg/kg s.c.) also attenuated cold allodynia in hind paw as compared to the vehicle-treated rats. Moreover, paclitaxel administration significantly increased the number of paw lifts in response to noxious cold stimuli as compared to the respective pre-paclitaxel baselines and naïve rats ($p<0.001$). Treatment with loperamide and gabapentin significantly decreased the number of paw lifts as compared to their pre-drug baselines and vehicle treated rats (Figure. 4.1D). These findings indicated that the peripheral MOR agonist is capable of reversing the mechanical and cold pain modalities in the CINP rats.

4.3.2 Activation of peripheral MOR attenuates ongoing pain in PTX-induced neuropathic rats

Spontaneous ongoing pain is among the most prominent symptoms of CINP. The effect of loperamide on spontaneous ongoing pain-like behaviour in CINP rats was assessed using the conditioned place preference (CPP) paradigm. Loperamide (20 mg/kg s.c.) significantly attenuated ongoing pain in PTX induced neuropathic rats as observed during the post conditioning trial where CINP rats showed increased preference ($p<0.01$) to the L20-paired chamber as compared to the vehicle paired chamber. Furthermore, there was a significant increase ($p<0.001$) in difference score in loperamide paired chamber as compared to the vehicle paired chamber in CINP rats. Further, post-conditioning trials in naïve rats revealed no significant preference for the loperamide chamber over the vehicle paired chamber, indicating that loperamide (20 mg/kg s.c.) did not induce place preference (Figure. 4.2 A-C, and 4.3). This suggests that loperamide induced CPP is due to its pain-relieving property and the molecule itself does not possess any abuse potential.

Similarly, we observed that gabapentin administration also significantly ($p<0.01$) increased place preference behaviour only in CINP rats and not in naive rats (Figure. 4.2 D-F, and 4.4).

Conversely, both CINP and naive rats treated with morphine showed significant ($p<0.001$) place preference behaviour, (Figure. 4.2. G-I, and 4.5) suggesting that the drug had both addictive and analgesic characteristics.

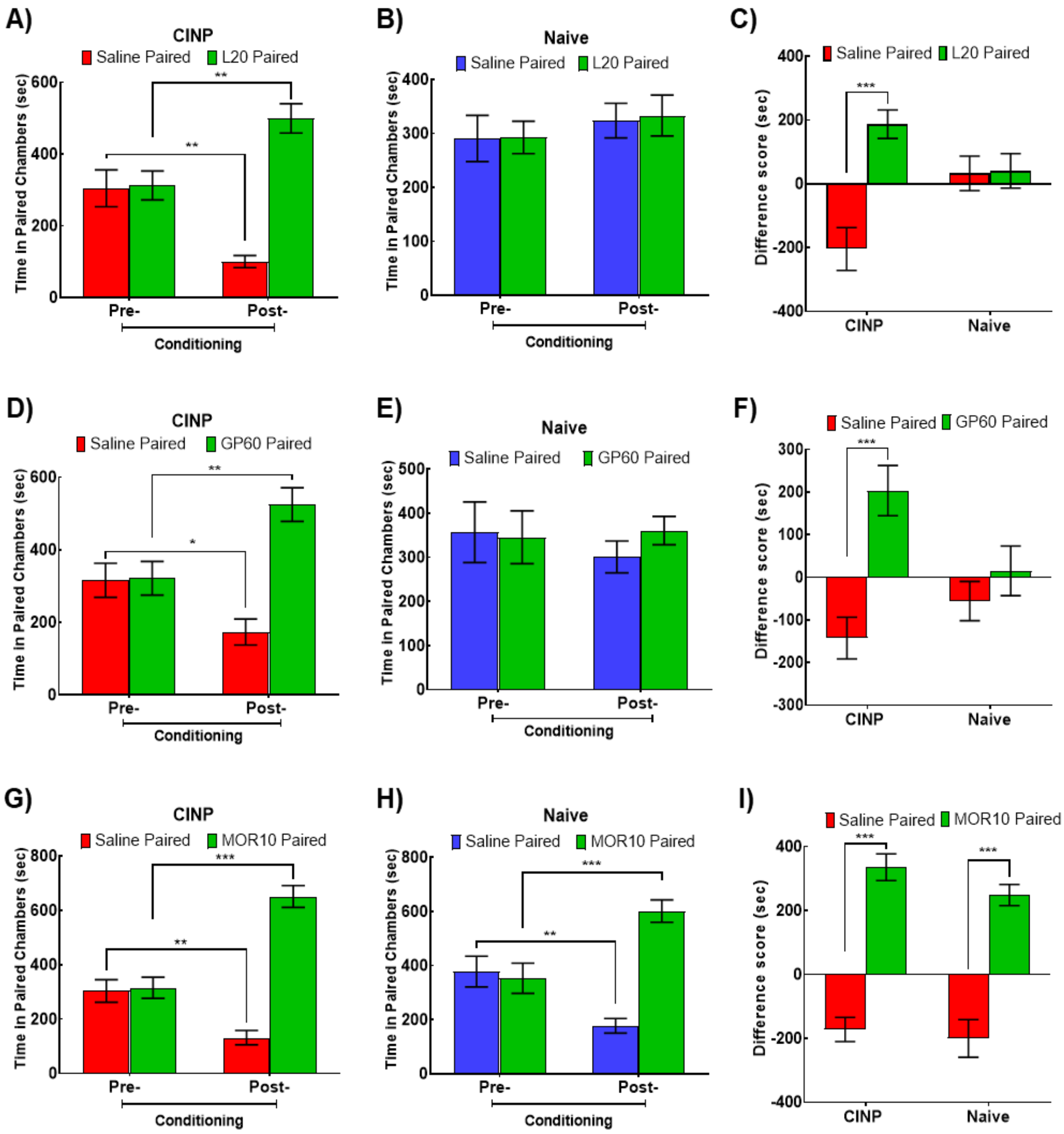

Figure. 4.2. Effect of peripheral MOR activation on CINP-induced spontaneous pain in rats. (A, B, C) Conditioned place preference (CPP) for loperamide in CINP rats, naïve rats & difference score: Neuropathic (PTX) rats showed significant place preference behaviour in response to loperamide (20mg/kg s.c.) treatment as indicated by increased time spent in loperamide paired chamber during post-conditioning. Notably, loperamide (20mg/kg s.c.) did not produce CPP in healthy naïve rats. **(D, E, F) CPP for Gabapentin in CINP rats, naïve rats & difference score:** Neuropathic (PTX) rats showed significant place preference behaviour to gabapentin (60mg/kg s.c.) during post-conditioning as compared to the pre-conditioning baseline. Interestingly, gabapentin (60mg/kg s.c.) treatment did not produce CPP in healthy naïve rats. **(G, H, I) CPP for morphine in CINP rats, naïve rats & difference score:** Paclitaxel-induced neuropathic rats showed significant place preference behaviour to morphine (10mg/kg s.c.) as indicated by increase preference to morphine paired chamber during post-conditioning as compared to the pre-conditioning baseline. However, naïve rats also

developed increased preference to morphine (10mg/kg s.c.) treatment, indicating both analgesic and addictive potential of morphine. Data was expressed as mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparison) (n=6-8). *($p<0.05$), **($p<0.01$), ***($p<0.001$) represents significant difference. Loperamide L20: 20mg/kg s.c., Gabapentin (GP60): 60mg/kg s.c., Morphine MOR10: 10mg/kg s.c.

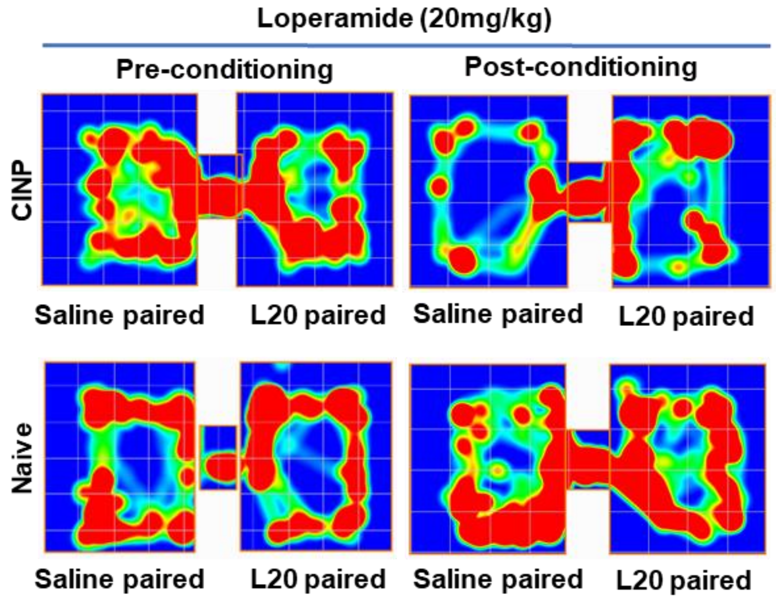

Figure 4.3 Effect of loperamide on CINP-induced spontaneous pain in rats. Figure shows heat maps recorded during pre-conditioning and post-conditioning with vehicle v/s loperamide (20mg/kg s.c) paired chambers.

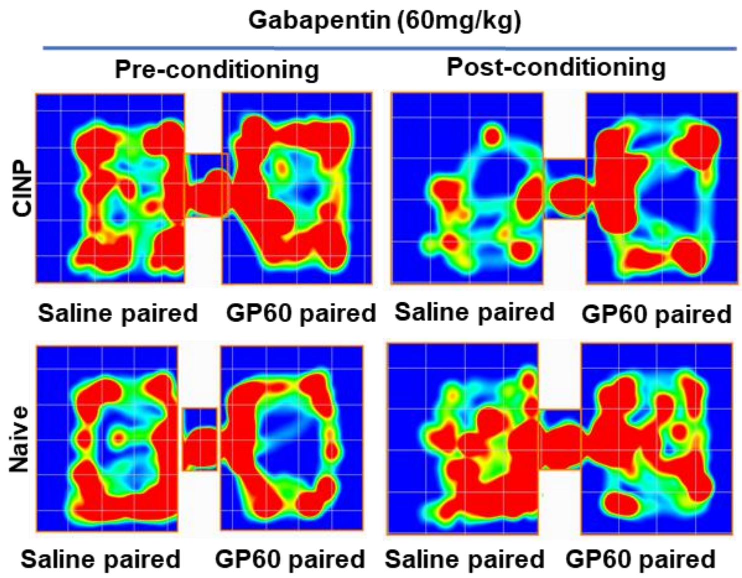

Figure. 4.4 Effect of gabapentin on CINP-induced spontaneous pain in rats. Figure shows heat maps recorded during pre-conditioning and post-conditioning with vehicle v/s gabapentin (60mg/kg s.c) paired chambers.

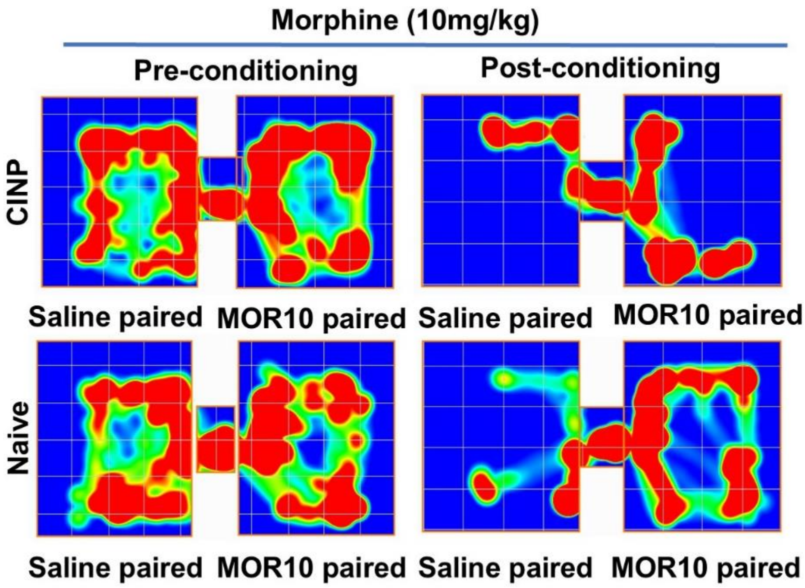

Figure. 4.5 Effect of morphine on CINP-induced spontaneous pain in rats. Figure shows heat maps recorded during pre-conditioning and post-conditioning with vehicle v/s morphine (10mg/kg s.c) paired chambers.

4.3.3 Loperamide treatment does not affect locomotor activity and motor coordination of rats

Although loperamide is a peripherally acting molecule, in this study we showed its efficacy in alleviation of spontaneous ongoing pain which is majorly regulated by central sensitization. Thus, we evaluated if loperamide is resulting in development of any central side effects. To assess the same, we have performed open field test and rota-rod test to measure the locomotor activity and motor coordination of rats. In open field test we have observed no difference in the average speed and total distance travelled by the rats after loperamide and gabapentin treatment. The CINP rats when treated with morphine, demonstrated its sedative property with a decrease in total distance travelled and average speed (Figure. 4.6 A-C).

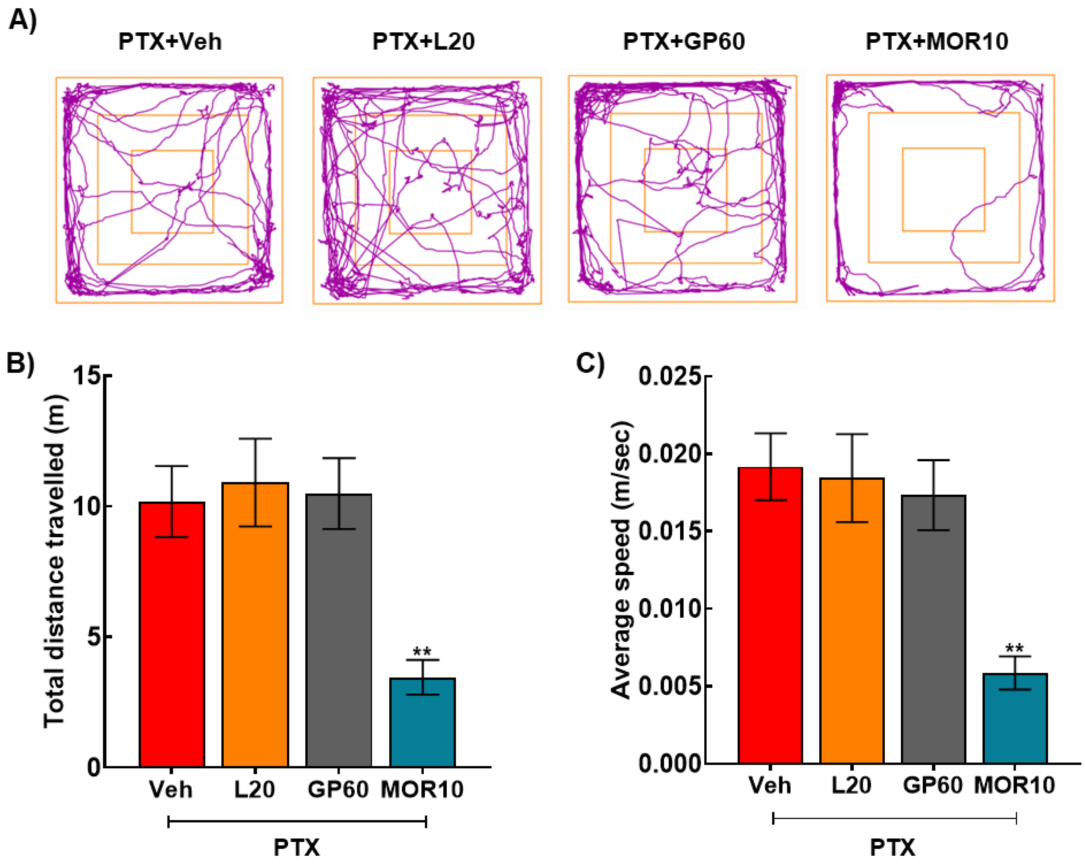

Figure. 4.6 Effect of peripheral MOR agonist, loperamide, on locomotor activity of rats. Open field test: (A) Open field track plots of CINP rats treated with vehicle, loperamide (20mg/kg s.c.), gabapentin (60mg/kg s.c.) and morphine (10mg/kg s.c.). **(B)** Total distance travelled in open field arena. **(C)** Average speed of rats treated with vehicle, loperamide (20mg/kg s.c.), gabapentin (60mg/kg s.c.) and morphine (10mg/kg s.c.). Loperamide (20mg/kg s.c.) and gabapentin (60mg/kg s.c.) treatment did not affect the locomotor activity of CINP rats in open field arena as compared to the vehicle treated rats. However, morphine (10mg/kg s.c.) treated rats showed a significant decline in total distance travelled and average speed. Data was expressed as Mean ± SEM and analyzed by using one-way ANOVA followed by Tukey’s post hoc analysis test (n=6-7). **$p<0.01$ indicates statistical significance as compared to the vehicle treated rats. Loperamide (L20): 20mg/kg s.c., Gabapentin (GP60): 60mg/kg s.c., Morphine (MOR10): 10mg/kg s.c.

In rotarod test, loperamide as well as gabapentin treatment demonstrated no significant difference in time spent on rota-rod compared to their pre-drug baselines. In contrast, morphine treatment revealed a substantial decrease in the fall latency in rats as compared to its pre-drug baseline, (Figure. 4.7) indicating its sedative effects.

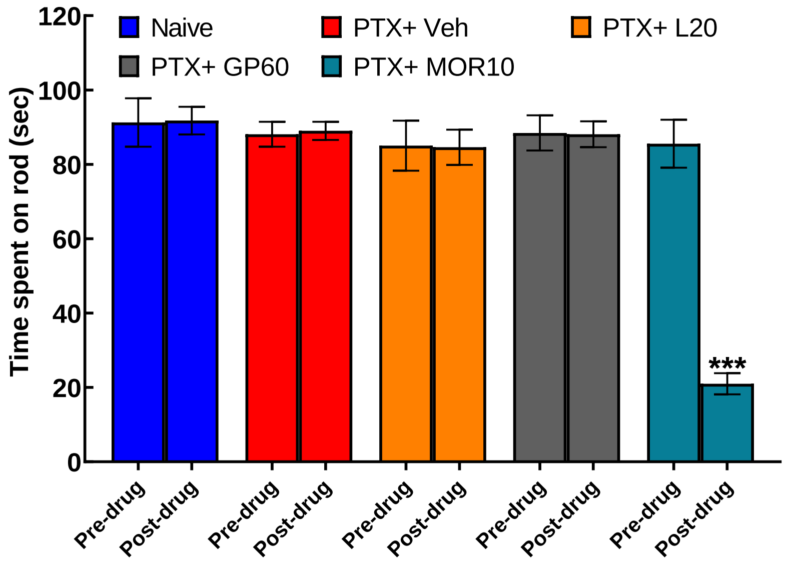

Figure 4.7 Effect of peripheral MOR agonist, loperamide, on motor coordination of rats. Rotarod test: Loperamide (20mg/kg s.c.) and gabapentin (60 mg/kg s.c.) treatment did not alter the time spent by rats on rota-rod as compared to their pre-drug baseline. However, morphine (10mg/kg s.c.) treatment significantly decreased the time spent by rats on rota-rod as compared to their pre-morphine baseline. Data was presented as mean ± SEM analyzed by two-way ANOVA (Bonferroni's multiple comparison) (n=6). ***$p<0.001$ indicates statistical significance as compared to the pre-drug baseline. Loperamide (L20): 20mg/kg s.c., Gabapentin (GP60): 60mg/kg s.c., Morphine (MOR10): 10mg/kg s.c.

4.3.4 Loperamide reversed oxido-nitrosative stress in sciatic nerve of PTX-induced neuropathic rats

Paclitaxel treatment resulted in development of oxido-nitrosative stress in the neuropathic rats. We observed that there is significant decrease in the anti-oxidant levels of GSH among all groups ($p<0.001$) and increase in reactive oxygen species that was indicated by increase in MDA (p<0.001) and nitrite levels (p<0.01). Upon treatment with loperamide the anti-oxidant levels were revived, which was observed with increase in the levels of GSH (5mg/kg, p<0.05; 10 and 20mg/kg, p<0.001) and oxidant levels were counteracted as the levels of MDA (10mg/kg, p<0.01, 20mg/kg, p<0.001) and nitrite (5mg/kg, p<0.05; 10mg/kg, p<0.01 and 20mg/kg, p<0.001) were reduced (Figure. 4.8 A-C) in the sciatic nerve of PTX induced neuropathic rats. Gabapentin treatment also improved the GSH levels (p<0.001) and reduced the levels of nitrite (p<0.001) and MDA (p<0.001) as compared to the vehicle treated CINP group.

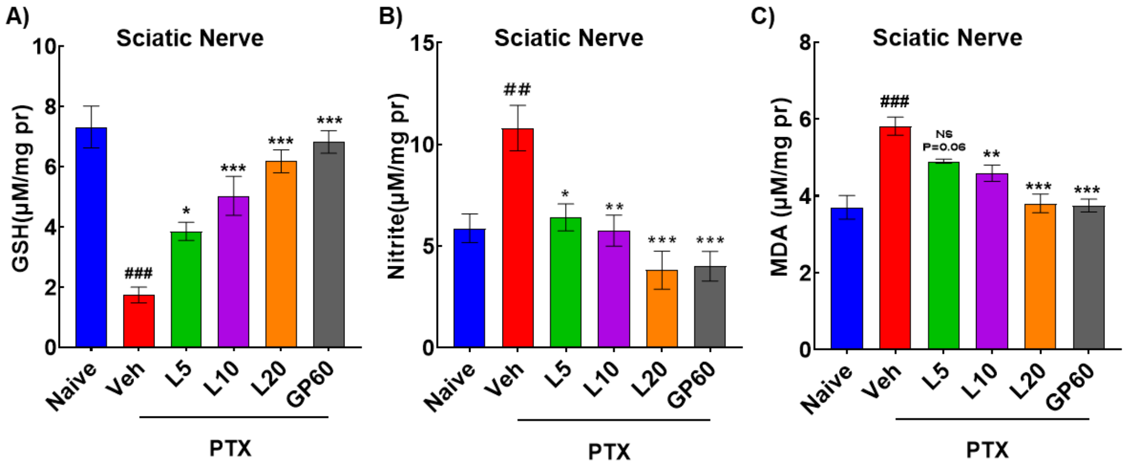

Figure 4.8 Effect of loperamide on CINP induced oxido-nitrosative stress in sciatic nerve of rats. (A) Nerve GSH: Paclitaxel administration resulted in decreased levels of antioxidant glutathione (GSH) in sciatic nerve of rats, which was significantly restored upon treatment with loperamide (5,10 & 20 mg/kg s.c.) and gabapentin (60mg/kg s.c.). **(B) Nerve nitrite and (C) Nerve malondialdehyde:** Paclitaxel administration resulted in increased nitrite and MDA levels in sciatic nerve of rats which was significantly decreased with loperamide (5,10 & 20 mg/kg s.c.) and gabapentin treatment (60mg/kg s.c.). Data was expressed as mean ± SEM (n=6-7). ## (p<0.01), ### (p<0.001) represents statistical significance compared to Naïve group. *(p<0.05), **(p<0.01), ***(p<0.001) represents statistical significance compared to PTX+ Vehicle group. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin (GP60): 60mg/kg.

4.3.5 Loperamide downregulates mRNA and protein expressions of VGSCs and TRP channels in the dorsal root ganglion of neuropathic rats

We have evaluated the alterations in expressions of voltage-gated sodium channels (VGSCs) and various TRP channels that play a key role in the development of paclitaxel induced neuropathic pain and subsequently the effect of loperamide and gabapentin on the same. In paclitaxel induced neuropathic pain the VGSCs (Nav1.7 & Nav1.8) are reportedly upregulated and play a crucial role in the somatosensory signal transmission in DRG [15,127]. To begin with VGSCs we observed that protein expression of Nav1.7 and mRNA expression of Nav1.8, were upregulated in the DRG of vehicle treated group ($F (5, 18) = 9.32$, $F (5, 18) = 8.64$) as compared to naïve group ($p<0.001$). Further we also observed that protein expression of TRPA1 and mRNA expression of TRPM8, were upregulated in the DRG of vehicle treated group ($F (5, 12) = 13.0$, $F (5, 18) = 7.53$) as compared to the naïve group ($p<0.001$). After loperamide treatment we found a significant reduction in protein and mRNA expressions of these key ion channels responsible for carrying out the peripheral sensitization in the DRG of neuropathic rats. Loperamide treatment decreased levels of Nav1.7 (5 and 10mg/kg; $p<0.05$, 20mg/kg; $p<0.01$), Nav1.8 (5mg/kg; $p<0.05$, 10mg/kg; $p<0.01$ and 20mg/kg; $p<0.001$), and TRPA1 (10mg/kg; $p<0.05$ and 20mg/kg; $p<0.01$), TRPM8 (5 and 10mg/kg; $p<0.05$ and 20mg/kg; $p<0.01$) in L4-L5 DRG tissues, which depicts its interference with the nociceptive transmission peripherally (Figure. 4.9 A-D). Gabapentin treatment also reduced the expressions of Nav1.7 ($p< 0.001$), Nav1.8 ($p < 0.01$), TRPA1 ($p<0.01$), TRPM8 ($p<0.01$) in L4-L5 DRG tissues.

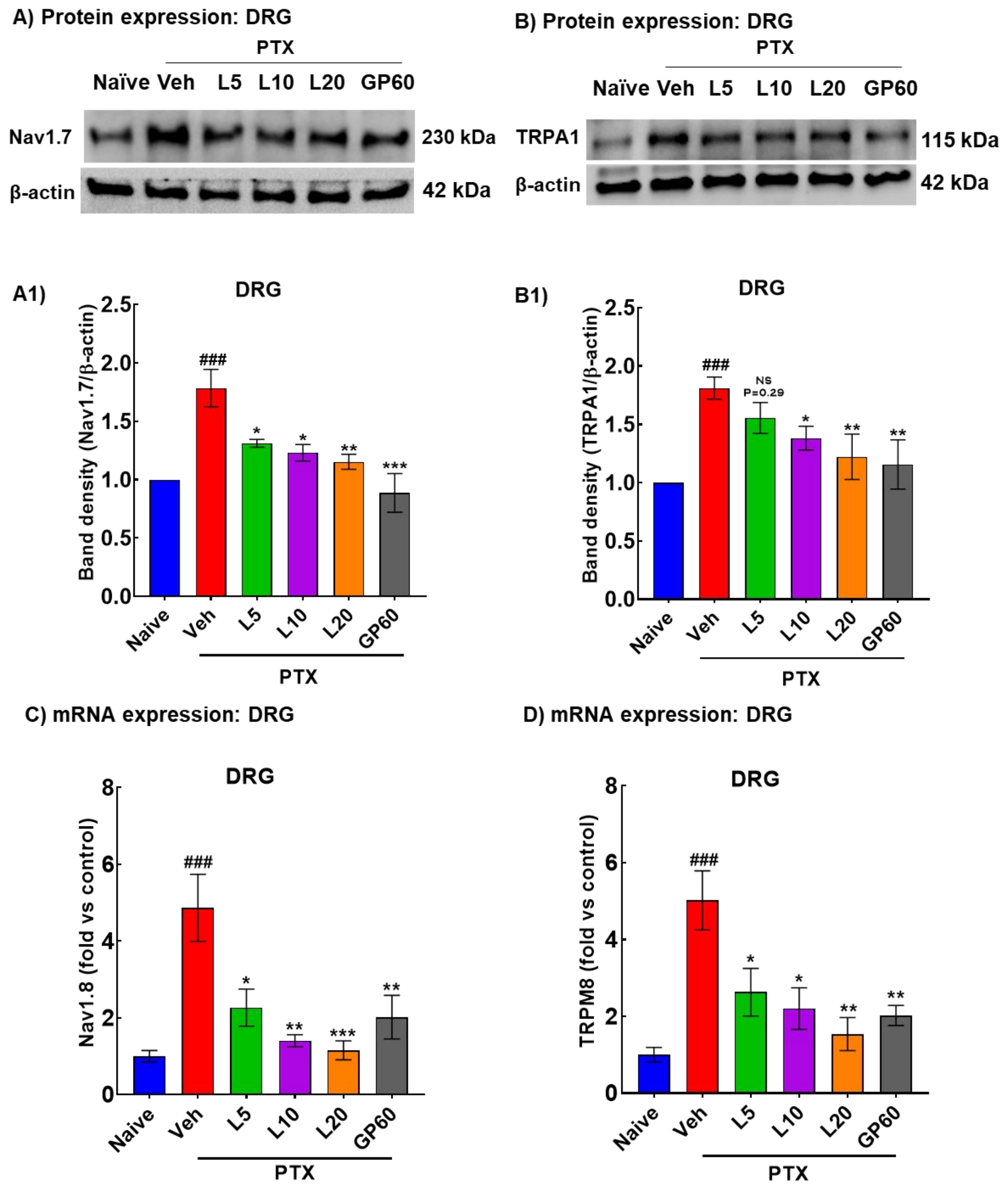

Figure 4.9 Effect of loperamide on CINP-induced increase in voltage gated sodium and TRP channels in lumbar DRG of rats. (A) Nav1.7 blot: Representative blots of Nav1.7 protein expression in DRG tissues of neuropathic rats. **(A1) Nav1.7 protein expression:** Paclitaxel administration increased the Nav1.7 protein expression in DRG of rats which was attenuated by the loperamide treatment (5, 10 & 20 mg/kg s.c.). **(B) TRPA1 blot:** Representative blots of TRPA1 protein expression in DRG tissues. **(B1) TRPA1 protein expression:** Loperamide (10 & 20 mg/kg s.c.) treatment significantly reversed paclitaxel-induced increase in TRPA1 expression in DRG of rats. Gabapentin treatment also showed decreased protein expression of both Nav1.7 and TRPA1 in DRG of neuropathic rats. **(C) Nav1.8 mRNA expression:** Paclitaxel administration

increased the Nav1.8 mRNA expression in DRG of rats which was attenuated by loperamide treatment. **(D) TRPM8 mRNA expression:** Increased mRNA expression of TRPM8 was significantly attenuated by loperamide (5,10 & 20 mg/kg s.c.) and gabapentin (60 mg/kg s.c.) treatment in DRG of neuropathic rats. Data was presented as mean ± SEM. (n=4). ### ($p<0.001$) represents statistical significance compared to Naïve group. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$ indicates statistical significance as compared to the PTX+ Veh rats. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin (GP60): 60mg/kg.

4.3.6 Loperamide attenuates neuropathic pain in rats via NR2B independent mechanism

As N-methyl-D-aspartate receptor (NMDAR) is one of the key regulators involved in chronic pain, we have evaluated the alterations in protein expression of its subunit NR2B in both DRG and spinal cord of CINP rats. PTX administration leads to significant upregulation in NR2B expressions in the DRG ($p<0.01$) with no changes in spinal NR2B expressions of rats as compared to the naïve group.

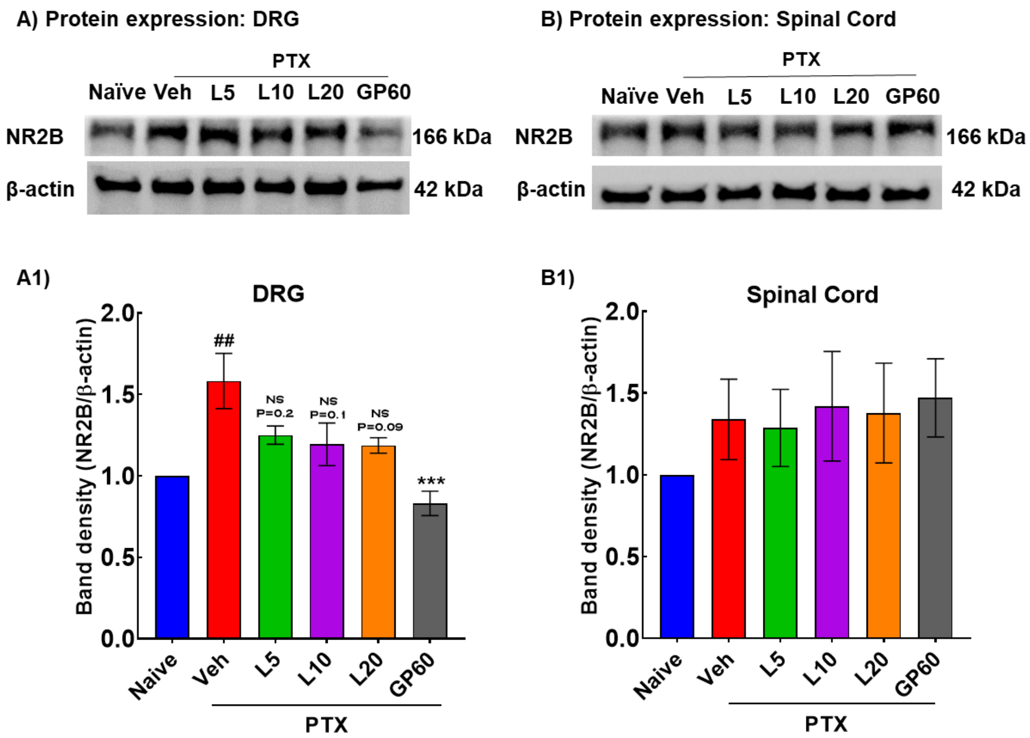

Figure 4.10 Effect of peripheral MOR activation on PTX-induced increase in NR2B protein expressions in DRG and spinal cord of rats. (A) NR2B blot (DRG):

Representative blots of NR2B protein expression in DRG of neuropathic rats. **(A1) NR2B protein expression (DRG):** Paclitaxel administration significantly upregulates NR2B protein expression in L4-L5 DRG of CINP rats which was downregulated by gabapentin (60mg/kg s.c.) treatment. Interestingly, loperamide treatment did not have any effect on PTX-induced NR2B protein expression. **(B) NR2B blot (spinal cord):** Representative blots of NR2B protein expression in spinal cord of neuropathic rats. **(B1) NR2B protein expression (spinal cord):** Paclitaxel administration did not alter NR2B protein expressions in spinal cord of rats which further remain unaffected on treatment with loperamide (5,10 & 20 mg/kg s.c.) and gabapentin (60 mg/kg s.c). Data was presented as mean ± SEM. (n=4). ## ($p<0.01$) represents statistical significance compared to Naïve group ***$p < 0.001$ indicates statistical significance as compared to the PTX+ Veh. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin (GP60): 60mg/kg.

Interestingly, loperamide treatment does not have any effect on upregulated NR2B in DRG of neuropathic rats. Moreover, gabapentin treated rats showed significant decrease in NR2B upregulation in DRG tissue of CIPN rats ($p<0.001$) (Figure. 4.10 A-B).

4.3.7 Loperamide suppressed expression of ICAM1 and CGRP but not IBA1 in spinal cord of CINP rats

Following paclitaxel exposure, there is disruption of vascular permeability of spinal cord, observed with increase in cell adhesion molecules (ICAM1) and simultaneous glial cell activation, indicated by increased expression of IBA1. We found a significant increase in spinal protein expression of ICAM1 and Iba1 ($F (5, 12) = 7.74$; $p=0.002$, $F (5, 12) = 5.643$; $p=0.007$) in vehicle treated PTX group as compared to naïve group. Although loperamide treatment was found to significantly decrease the expression of ICAM1 (10mg/kg; $p<0.05$ and 20mg/kg; $p<0.01$) it did not alter the protein levels of IBA1 in spinal cord of neuropathic rats, indicating its microglia independent mechanism. Gabapentin decreased the protein expression of ICAM1 ($p<0.01$), and IBA1 ($p<0.05$) in spinal cord of PTX induced neuropathic rats (Figure. 4.11A).

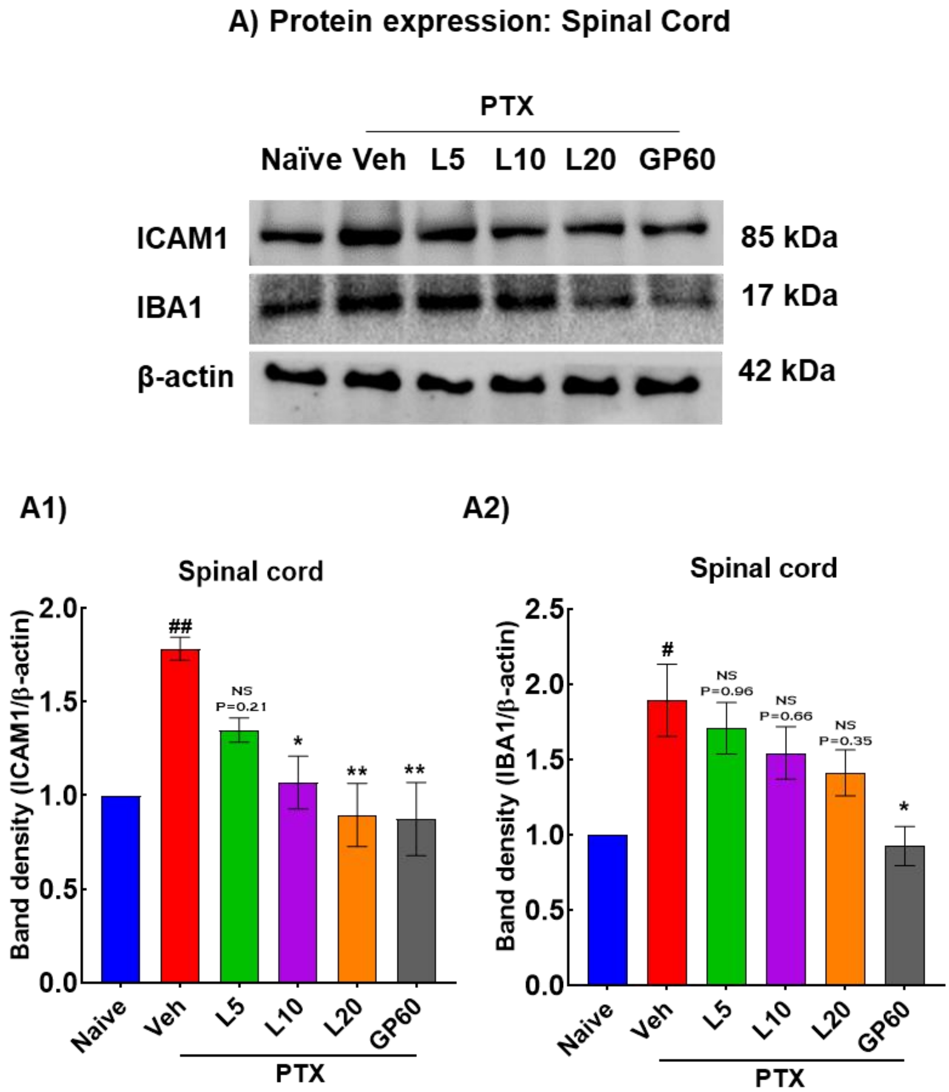

Figure 4.11 Effect of loperamide on CINP-induced increase in ICAM1, IBA1 in lumbar spinal cord of rats. (A) ICAM1 and IBA1 blots: Representative blots of ICAM1 and IBA1 protein expressions in spinal cord tissues. **(A1) ICAM1 protein expression:** Paclitaxel treatment increased the ICAM1 protein expression in spinal cord of rats which was attenuated by loperamide treatment (10 & 20 mg/kg s.c.) **(A2) IBA1 protein expression:** Paclitaxel administration increased the IBA1 protein expression in spinal cord of rats. However, loperamide does not have any effect on its expression unlike gabapentin, that significantly reduced the expression of IBA1. Data was presented as mean ± SEM. (n=4), #(p<0.05), ##p<0.01 represents statistical significance compared to Naïve group. *p < 0.05, **p < 0.01 indicates statistical significance as compared to the PTX+ Veh rats. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin (GP60): 60mg/kg.

Further, we also evaluated the mRNA expressions of the CGRP and found a significant upregulation in spinal cord of vehicle treated group ($F (5, 12) = 7.40$; $p=0.002$) as compared to naïve group. Treatment with both loperamide and gabapentin significantly downregulates CINP-induced spinal CGRP expressions of rats (10 and 20mg/kg; $p<0.05$) (Figure. 4.12).

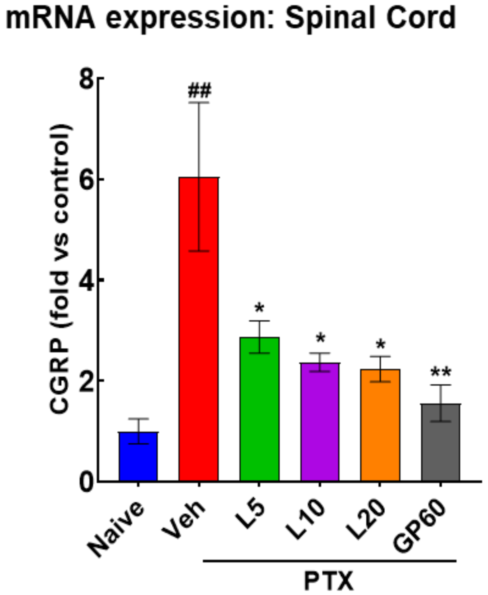

Figure 4.12 Effect of loperamide on CINP-induced increase in CGRP in lumbar spinal cord of rats. CGRP mRNA expression: Treatment with paclitaxel increased the mRNA expression of CGRP in spinal cord of rats which was attenuated by treatment with loperamide (5, 10 & 20 mg/kg s.c.) and gabapentin (60 mg/kg s.c.). Data was presented as mean ± SEM. (n=4), ##$p<0.01$ represents statistical significance compared to Naïve group. *$p < 0.05$, **$p < 0.01$ indicates statistical significance as compared to the PTX+ Veh rats. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin (GP60): 60mg/kg.

4.3.8 Loperamide attenuates central sensitization by modulating inflammatory signalling in DRG and spinal cord of PTX induced neuropathic rats

Reportedly paclitaxel administration causes increased expression of nuclear factor- κβ (NF-κβ) which further regulates and triggers release of various pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [128,129]. Neuropathic rats showed a significant increase in protein expression of NF-κβ, TNF-α and IL-1β in lumbar spinal cord tissue ($F_{(5, 18)} = 6.709$; $p=0.001$, $F_{(5, 12)} = 6.73$; $p=0.003$, $F_{(5, 18)} = 5.958$; $p=0.002$) as compared to the naïve rats. Loperamide treatment significantly downregulates protein expressions of NFκβ (5 and 10mg/kg; $p<0.05$ and 20mg/kg; $p<0.01$), TNF-α (5 and 10mg/kg; $p<0.05$, 20mg/kg; $p<0.01$), and IL-1β (10 and 20mg/kg, $p<0.05$) indicating its potential to attenuate inflammatory signaling and central sensitization (Figure. 4.13A). The neuro-immune interactions cause release of pro-inflammatory mediators even in the DRG. We found a significant upregulation in TNF-α protein expression in L4-L5 DRG of CINP rats ($F_{(5, 18)} = 13.6$, $p<0.001$) which was significantly downregulated upon treatment with loperamide (5 and 10mg/kg, $p<0.05$; 20mg/kg, $p<0.01$) (Figure. 4.13B). Gabapentin treatment also downregulates the protein expression of NFκβ ($p<0.01$), TNF-α ($p<0.01$) and IL-1β ($p<0.05$) in the spinal cord and TNF-α ($p<0.001$) in DRG of PTX-induced neuropathic rats.

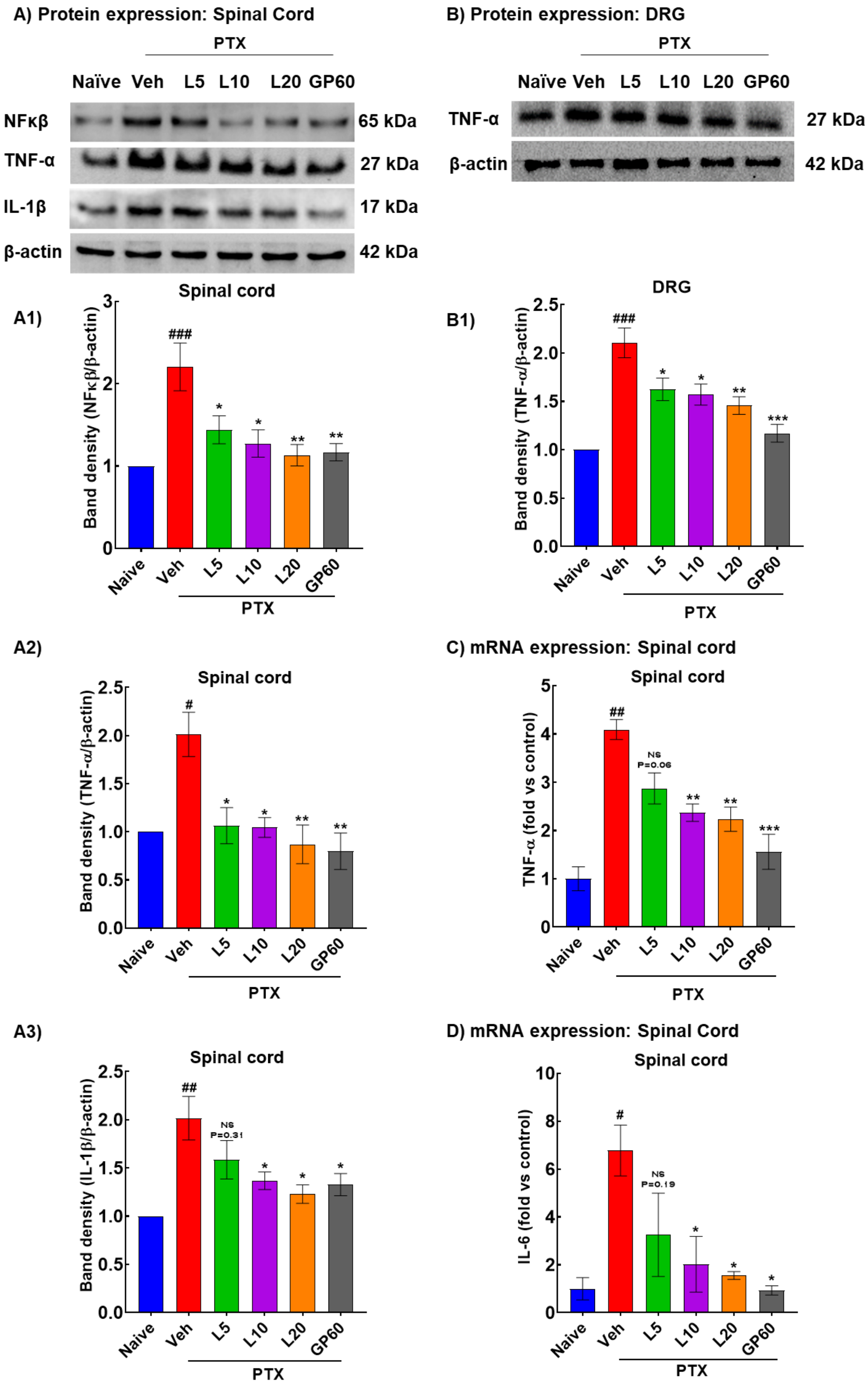

Figure 4.13 Effect of loperamide on CINP-induced nuclear factor-κβ activation and neuro-inflammation in DRG and spinal cord of rats. (A) NF-κβ, TNF-α & IL-1β blots (spinal cord): Representative blots of NFκβ, TNF-α, IL-1β protein

expressions in spinal cord of rats. **(A1) NF-κβ protein expression (spinal cord), (A2) TNF-α protein expression (spinal cord), and (A3) IL-1β protein expression (spinal cord):** Paclitaxel administration significantly increased the protein expressions of NF-κβ, TNF-α & IL-1β in spinal cord of rats which was attenuated by loperamide (5,10 & 20 mg/kg s.c.) and gabapentin (60mg/kg s.c.) treatment. **(B) TNF-α blots (DRG):** Representative blot of TNF-α protein expression in DRG of rats. **(B1) TNF-α protein expression (DRG):** Paclitaxel administration upregulates TNF-α protein expression in DRG of rats which was attenuated by the loperamide treatment (5,10 & 20 mg/kg s.c.). **(C) TNF-α mRNA expression (spinal cord), and (D) IL-6 mRNA expression (spinal cord):** Paclitaxel administration also upregulates mRNA expressions of TNF-α and IL-6 in spinal cord of rats which was significantly attenuated on treatment with loperamide (5,10 & 20 mg/kg s.c.) and gabapentin (60mg/kg s.c.). Data was presented as mean ± SEM. (n=4), #($p<0.05$), ##$p<0.01$, ###$p<0.001$ represents statistical significance compared to Naïve group. *$p < 0.05$, **$p < 0.01$ indicates statistical significance as compared to the PTX+ Veh rats. Loperamide doses: L5: 5mg/kg, L10: 10mg/kg, L20: 20mg/kg. Gabapentin GP60: 60mg/kg.

Further, we also evaluated the mRNA expressions of TNF-α and IL-6 in spinal cord tissues and found a significant upregulation in vehicle treated PTX group ($F (5, 12) = 15.9$; $p<0.001$, $F (5, 12) = 5.10$; $p=0.010$). Loperamide treatment significantly downregulates mRNA expression of TNF- α (10 and 20mg/kg; $p<0.01$) and IL-6 (10 and 20mg/kg; $p<0.05$) in spinal cord of CINP rats as compared to vehicle treated rats. Gabapentin treatment also decreased the mRNA expression of TNF- α ($p<0.001$) and IL-6 ($p<0.05$) in spinal cord of neuropathic rats (Figure. 4.13 C-D).

4.4 Outcomes

In conclusion, our findings suggest that activation of peripheral MOR by loperamide mitigates paclitaxel-induced pain-like behaviour in rats by downregulating TRP channels and VGSCs coupled with the inhibition of oxido-nitrosative and neuroinflammatory signaling.