CHAPTER 4: Development and Pharmacological Validation of Clinically Mimicable Animal Model of Combined Chemotherapy-Induced Neuropathic Pain

4.1 Introduction

Cancer continues to be a widespread and challenging disease, significantly impacting the quality of life and leading to mortality and morbidity worldwide. Chemotherapy stands as a prominent approach in combating for the treatment of cancer, but its therapeutic benefits are often accompanied by considerable costs. The off-target effects, dose-limiting toxicity and other severe side effects such as peripheral neuropathy, underscore the challenges associated with the treatment in clinics. CINP is a major complication of anti-cancer drugs, presenting a significant challenge in cancer treatment. This condition creates a considerable clinical dilemma, as it requires comprehensive management strategies to effectively alleviate pain and ensure patients can continue with their treatment for the best possible outcomes. The prevalence of CINP varies widely reported in the range of 18% to 88% among cancer patients undergoing treatment with various anticancer drugs (Baxi et al., 2020). CINP results from chemotherapy-induced damage to sensory nerves, setting off a chain of events involving free radical generation, mitochondrial dysfunction, activation of glial cells, and neuro-inflammation, coupled with ion channel activation. Ultimately, this cascade leads to the development and progression of neuropathic pain (De Logu et al., 2020)

TRP channels, including TRPA1, TRPV1, and TRPM8, have emerged as key players in the pain processing from peripheral to central site. The activation of these nociceptors further triggers the release of calcitonin gene-related peptide (CGRP) and substance P, thereby perpetuating neuro-inflammation and culminating in the

recruitment of immune cells, notably T cells (Ullah et al., 2021). Moreover, accumulating evidence points to the involvement of N-methyl-D-aspartate receptors (NMDARs), particularly those containing the NR2B subunit, in the initiation and maintenance of CINP by facilitating central sensitization (Y. Y. Zhang et al., 2022). Unfortunately, there are currently no effective drugs available for the treatment of CINP, and existing medications for neuropathic pain are inadequate due to its complex pathophysiology. Therefore, there is an urgent need to develop better therapeutics for the same.

Despite vast investigation on drug development for the CINP, the overall success rate of drug translation to the clinical setup remains low due to several factors. One such factor is the lack of animal models which can mimic the clinical setup of chemotherapy treatment and correlate the outcome to bridge the translational gap. Paclitaxel/platinum/vincristine combinations delivered significant outcomes in phase III clinical trials and are now considered standard therapy for advanced carcinomas including breast cancer and gastrointestinal cancers (Magge and DeAngelis 2015; Amini et al. 2016; Pollard et al. 2021). For several decades' the mono-chemotherapy model of CIPN is being used by researchers for investigating pathophysiology and screening novel analgesic therapies. However, in the clinic, antineoplastic agents are prescribed in combination with two or three drugs for the treatment of cancer (Lane, 2006; Li et al., 2014; Phua et al., 2019). Given the widespread use of this clinical approach, there is an unmet need for a research model that mirrors the complexity of combination chemotherapy. Recognizing the prevalence of combination chemotherapy in clinical settings, our study aims to fill this significant gap in CINP research.

We have developed a rat model for CINP grounded in a combination-based approach and our aim was to create an alternative animal model that faithfully recapitulated the clinical complexity of CINP, providing a more clinically relevant platform for scientific investigation and therapeutic development. To achieve this, we adopted a rigorous three-step research strategy that incorporated behavioral, pharmacological, and molecular tools. Our model aims to capture the essence of CINP, ensuring that the observed manifestations closely resemble the clinical scenario. Predictive validity we rigorously assess the model's ability to respond to standard therapeutic interventions employed in clinical practice. Constructive validity is the third dimension of our research strategy delves into the biological changes induced by our model and compares them to those encountered in patients with CINP. This constructive validity is critical for establishing a degree of concordance between the molecular, cellular, and physiological alterations induced by our model and those observed in clinical CINP.

4.2 Experimental procedure

In this study, our experiments were divided into two distinct phases, each serving a specific research objective. The initial phase aimed to characterize the time course of pain responses induced by various chemotherapeutic agents. Three groups of rats, each comprising 8-9 animals, were established. Group 1 received a vehicle solution consisting of 4% DMSO in sterile saline, while Group 2 was administered paclitaxel (PTX) at a dose of 2 mg/kg intraperitoneally (i.p.). Group 3 received a combination chemotherapy regimen comprising paclitaxel (2 mg/kg), cisplatin (2 mg/kg), and vincristine (0.5 mg/kg), all delivered via intraperitoneal injection. Baseline were recorded before administering the chemotherapeutic agents, which were prepared by

dissolving them in a 4% DMSO solution. Subsequently, these solutions were intraperitoneally administered on four alternate days (0, 2, 4, and 6). Alongside we have performed behavioral assessments, to evaluate pain intensity and pain persistence, as well as we have also checked the cellular and molecular alterations in the dorsal root ganglion (DRG), the lumbar region of the spinal cord, and the sciatic nerve. In the second phase of the study, we focused on assessing the pharmacological validation of two distinct models of CINP and investigating the potential therapeutic effects of gabapentin as standard drug. Rats were divided into five groups, each comprising eight animals. Group 1 received the same vehicle solution of 4% DMSO in sterile saline, while Group 2 received paclitaxel (PTX) at a dose of 2 mg/kg i.p. Group 3 received a combination chemotherapy regimen consisting of paclitaxel (2 mg/kg), cisplatin (2 mg/kg), and vincristine (0.5 mg/kg) administered via intraperitoneal injection. In Group 4, rats were administered PTX (2 mg/kg) along with gabapentin (60 mg/kg), both intraperitoneally. Group 5 received the combined chemotherapy regimen in conjunction with gabapentin (60 mg/kg, i.p.). Baseline were recorded for all five groups before chemotherapeutic administration, followed by intraperitoneal administration of PTX and the combined chemotherapy regimen over four alternate days (0, 2, 4, and 6). For Groups 4 and 5, designated as the standard groups, gabapentin was administered intraperitoneally once daily from day 1 to day 8, concurrent with chemotherapy administration. Subsequent to drug administration, behavioral and molecular studies were conducted to assess whether gabapentin had mitigated pain hypersensitivity and influenced biological alterations resulting from chemotherapy exposure.

4.3 Results and discussion

4.3.1 Effect of combined chemotherapy on daily body weight changes in rats

In our study, we closely monitored daily body weight changes and key clinical signs such as alopecia, diarrhoea, and digestive discomfort in rats undergoing chemotherapy. We used a combination of potent drugs (paclitaxel, cisplatin, vincristine) to mimic real-world cancer treatment. During the early chemotherapy phase, we observed a rapid drop in rat body weight, consistent with previous research. Notably, paclitaxel and cisplatin were found to induce quick weight loss shortly after treatment initiation. This acute weight loss is linked to chemotherapy-related digestive issues, nausea, vomiting, and reduced food intake, highlighting the importance of caloric intake in understanding body weight changes during treatment (Höke and Ray, 2014). However, we did not observe any signs of alopecia or diarrhoea in the animals during entire study. Moreover, although there was one instance of mortality recorded within the combined chemotherapy-treated group, no such mortality was observed in the rats administered with paclitaxel (PTX).

To systematically monitor body weight changes, rats were weighed prior to chemotherapy initiation and baseline values was recorded to the animals. Subsequently on days 2, 4, 8, 14, 21, 28, 35, 42, 49, and 56 following the last chemotherapy injection. Evidently, there was a significant reduction in body weight of combined chemotherapy administered rats as compared to their corresponding initial body weight, the PTX-administered group, and the vehicle-treated group. Further, the PTX and vehicle-treated rats gained normal weight simultaneously throughout the course of the study. Using repeated measures Two-way ANOVA analysis revealed a significant effect across the groups [$F_{19,25}=6.25$; $p<0.01$]. In contrast, PTX-administered rats and vehicle-treated

group exhibited no significant variance in body weight throughout the duration of the study, as demonstrated in **Figure 4.1** (n=8 per group). This comprehensive evaluation of clinical observation and body weight dynamics in the context of combined chemotherapy underscores its paramount significance as an indicator of overall health and tolerance to treatment.

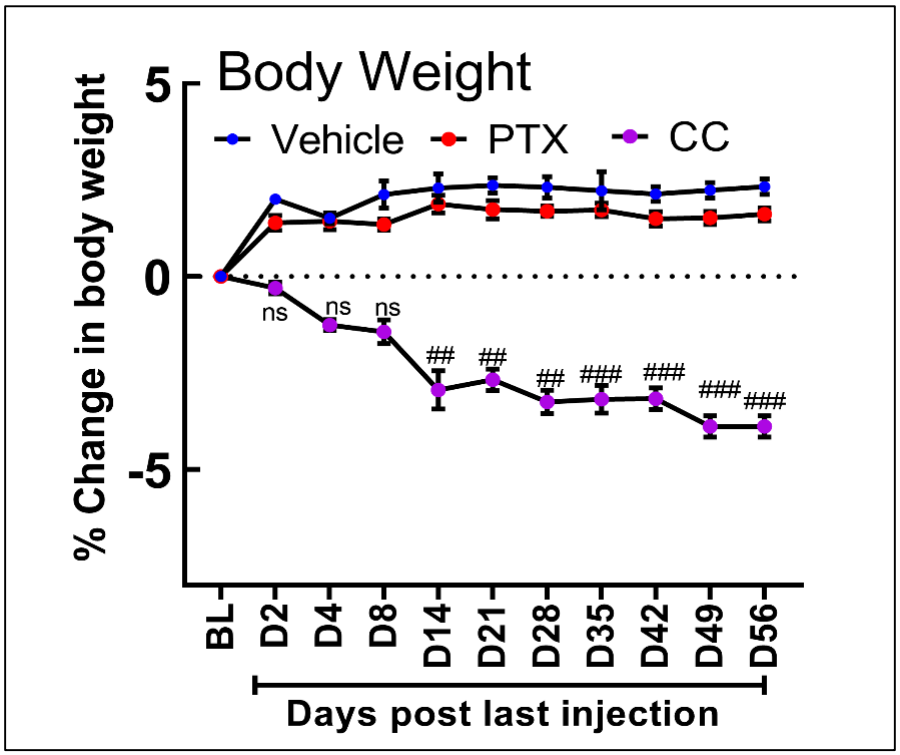

Figure 4.1 Effect of combination chemotherapy on body weight of rats. % body weight change of paclitaxel and combined chemotherapy treated rats. The rats were weighed on day 0 (BL) and on day 2, 4, 8,14, 21, 28, 35, 42,49 and day 56 post-last chemotherapy administration. The results of all groups were compared with each other and their respective baseline. Rats that received combined chemotherapy had significantly decreased body weight as compared to the PTX and vehicle-treated groups. However, PTX and vehicle-treated rats did not show any significant difference in their body weights at any time point during the study. Data were presented as mean ± SEM, $^{###}P<0.001$, $^{##}P<0.01$ indicates statistical significance as compared to the relative baseline. $P<0.05$ was considered statistically significant.

4.3.2 Combination chemotherapy causes pain like behaviours in rats

4.3.2.1 Combination chemotherapy causes thermal and mechanical hypersensitivity in rats

Numerous studies utilizing rat models have consistently demonstrated the development of most prominent pain like symptoms such as thermal hyperalgesia, mechanical allodynia and cold hyperalgesia in response to chemotherapeutic agents. Instance, paclitaxel, a commonly used chemotherapeutic drug, has been shown to induce robust thermal hyperalgesia and mechanical allodynia in rats (Boehmerle et al., 2014). Most importantly the use of chemotherapy leads to the damage across the somatosensory nervous system thus precipitates CINP. In our study, we conducted a series of pain behavioural assessments aimed at evaluating neuropathic pain in rats subjected to combined chemotherapy. These assessments involved exposing the animals to both thermal and mechanical stimuli, allowing us to gauge their pain responses. Importantly, we have measured the baseline pain thresholds of animals before the administration of the first dose of paclitaxel (PTX) and combined chemotherapy regimen. We continued to monitor and assess the rats' responses for a duration of 56 days following the last injection of chemotherapy.

Our findings revealed significant alterations in thermal and mechanical sensitivity in rats subjected to PTX and combined chemotherapy. Notably, on day 4 following the last injection of PTX and combined chemotherapy, rats exhibited a substantial increase in thermal and mechanical hypersensitivity, indicative of heightened pain responses. The peak of this hypersensitivity was observed on day 14, emphasizing the acute and intense nature of the pain induced by these treatments (**Figure 4.2 n=8; p<0.01**). In the combined chemotherapy-treated group, thermal and

mechanical hypersensitivity displayed a remarkable persistence, extending into a long-lasting plateau phase that persisted for up to day 56 following the final combined chemotherapy injection. In contrast, in the PTX-treated group, the thermal and mechanical hypersensitivity lasted up to day 35 following the fourth PTX injection **(Figure 4.2 A-D; n=8; p<0.01).** Using repeated-measures two-way ANOVA revealed significant effects across the groups and interactions, underscoring the substantial effect of the chemotherapy regimens on paw withdrawal latency (PWL) and paw withdrawal threshold (PWT) ($p<0.001$). These findings provide compelling evidence of the persistence and intensity of neuropathic pain induced by combined chemotherapy, as well as its distinct temporal profile compared to PTX-induced pain. Remarkably, our study also demonstrated that thermal and mechanical hypersensitivity gradually returned to normal levels in PTX-treated rats by day 35 and in combined chemotherapy-administered rats by day 56 post last injection.

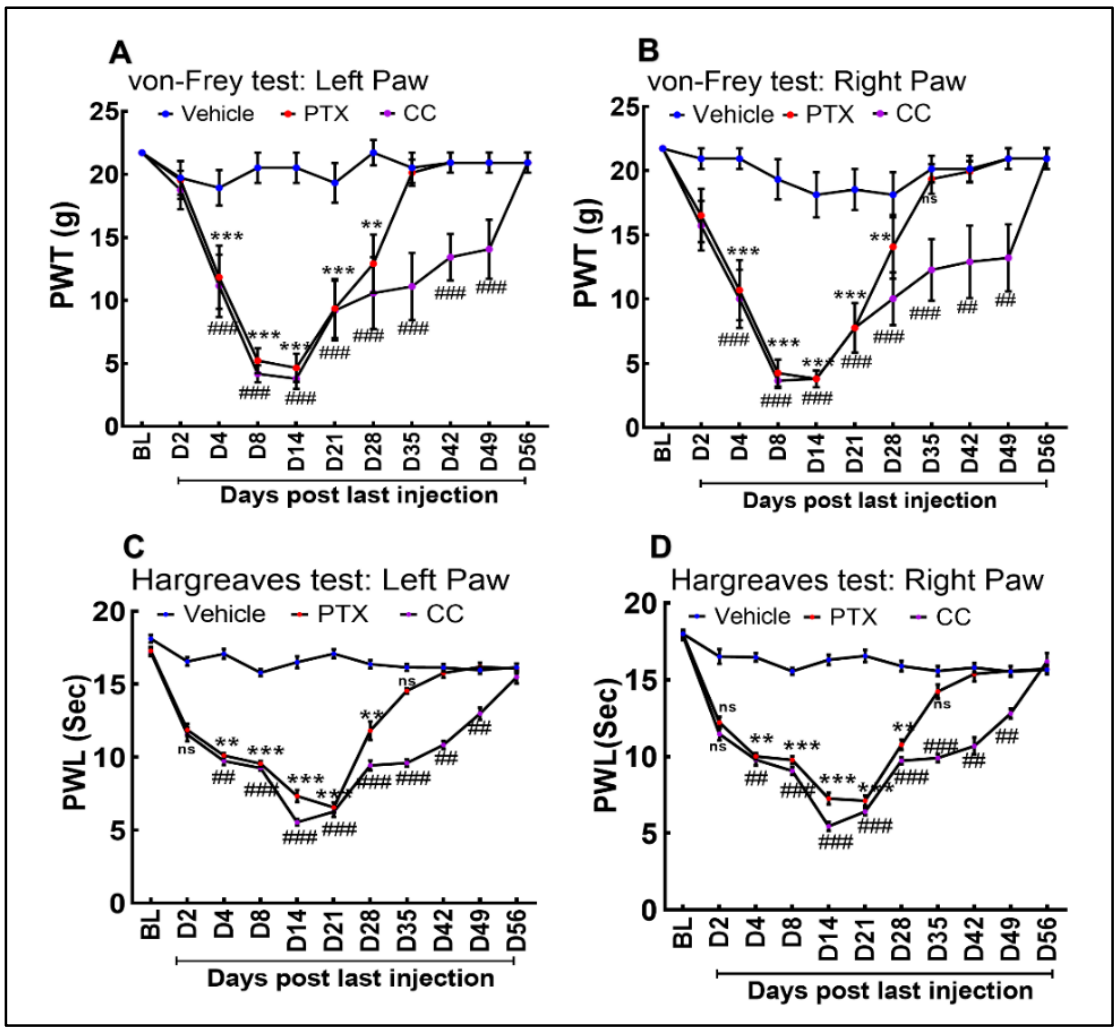

Figure 4.2 Combination chemotherapy induces persistent pain behavior in rats. Combined chemotherapy (CC) administration led to significant and time-dependent development of thermal hyperalgesia, mechanical allodynia, cold allodynia, and cold hyperalgesia in rats from day 4 to 49. Pain sensitivity returned to pre-chemo baseline in CC treated rats on day 56 post-last CC injection. However, paclitaxel (PTX) treated rats showed increased pain response from days 4 to day 28 post last injection of PTX and reversal to pre-PTX baseline was observed at day 35. **von Frey hair test (A)** paw withdrawal threshold in left hind paw. **B)** paw withdrawal threshold in right hind paw. **Hargreaves test (C)** paw withdrawal latency in left hind paw **(D)** paw withdrawal latency in right hind paw. Results were expressed as mean ± SEM. ***p<0.001 and **p < 0.01 comparison between PTX group and vehicle-treated group. ###p < 0.001 and##p < 0.01 comparison between CC group with PTX and vehicle-treated groups. n=8 rats/group.

4.3.2.2 Combined chemotherapy develops cold hypersensitivity in rats

Numerous preclinical investigations using rodent models have yielded compelling evidence supporting the existence of cold allodynia and cold hyperalgesia in the context of CINP induced by paclitaxel (PTX) and cisplatin. These animal models, when exposed to PTX and cisplatin, consistently exhibit heightened sensitivity to innocuous cold stimuli, which is indicative of cold allodynia (Polomano et al., 2001). Furthermore, exposure to noxious cold stimuli results in intensified pain responses in these animals, effectively mirroring the clinical presentation of cold hyperalgesia observed in patients undergoing chemotherapy (Toma et al., 2017). Clinical assessments, involving standardized pain scales and sensory testing, have provided quantitative evidence reaffirming the prevalence of cold allodynia and cold hyperalgesia in CINP. In our experimental observations, we noted a significant increase in chemotherapy-induced pain intensity on day 4, which persisted up to day 56 in combined chemotherapy model and day 35 in the PTX model following the last injection of anticancer agents, as compared to the vehicle-treated groups (**Figure 4.3 A and B; n=8 per group; $p<0.05$**). This was determined through two-way ANOVA followed by Bonferroni's multiple comparison test. To evaluate non-noxious stimuli evoking cold allodynia in neuropathic rats, we employed the acetone drop test. Repeated-measures two-way ANOVA followed by Bonferroni's multiple comparison tests suggest a significant effect across the groups' interaction [$F_{18,212}=36.2$; $p<0.001$] and [$F_{18,215}=27.20$; $p<0.001$] in the response score of vehicle-treated rats, PTX-treated rats, and combined chemotherapy-treated rats. Both PTX and combined chemotherapy administrations significantly increased paw withdrawal responses, indicative of cold-evoked pain behaviours in both the left and right hind paws when

compared to their respective pre-chemotherapy baselines and vehicle-treated rats ($p<0.001$) (**Figure 4.3 C and D; n=8 per group; $p<0.05$**). These findings collectively suggest that the combined chemotherapy model induces a more prolonged and sustained pain behaviour in rats compared to the PTX model.

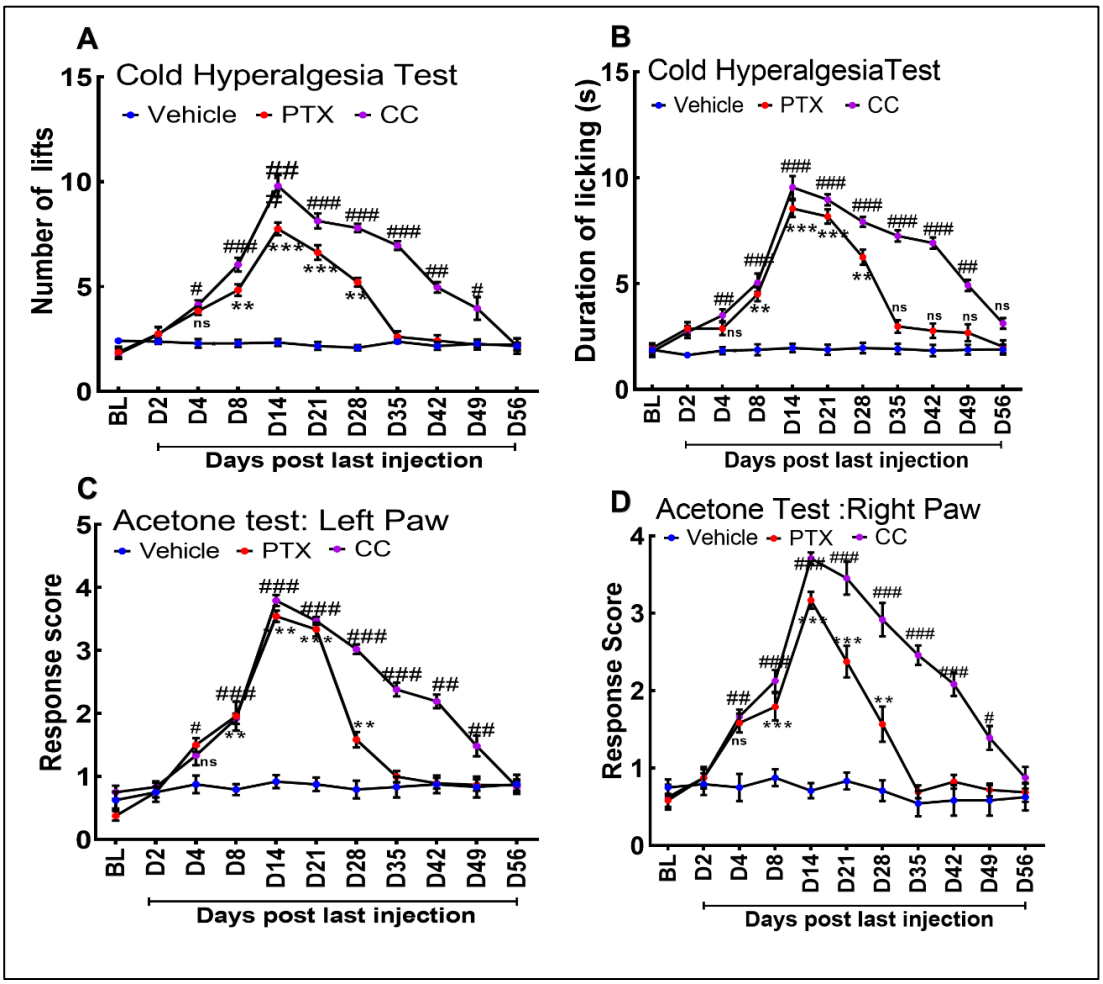

Figure 4.3 Combination chemotherapy induces cold hypersensitivity in rats. Combined chemotherapy (CC) administration led to significant and time-dependent development of cold hyperalgesia and cold allodynia in rats from day 4 to 49. **Cold hyperalgesia test (A)** number of paw lifts or paw licks in response to noxious cold stimuli **(B)** duration of paw licking in response to noxious cold stimuli. **Acetone test (C)** paw withdrawal response in left hind paw **(D)** paw withdrawal response in right hind paw. Results were expressed as mean ± SEM. ***p<0.001 and **p < 0.01 comparison between PTX group and vehicle-treated group. ###p < 0.001 and ##p < 0.01 comparison between CC group with PTX and vehicle-treated groups. n=8 rats/group.

4.3.3 Modulation of TRP channels, NR2B, neuropeptides and inflammatory signaling in DRG and Spinal cord of neuropathic rats

4.3.3.1 Combined chemotherapy upregulates TRP Channels mRNA expression in DRG and spinal cord of rats.

TRP channels, specifically TRPV1, TRPA1, and TRPM8, play pivotal roles in sensing a wide range of temperature thresholds, spanning from cold sensations to noxious heat perception (Chukyo et al., 2018; Khan et al., 2021). These specialized nociceptors are intricately involved in the pathophysiology of CINP by facilitating the influx of intracellular calcium ions (Ca^{2+}), which in turn modulates various downstream signalling pathways. TRPV1 is renowned for its role in responding to noxious heat, making it a key player in the transmission of pain signals in response to high temperatures (Yang et al., 2010; Zhai et al., 2020). TRPA1, on the other hand, is activated by various noxious stimuli, including cold temperatures and chemical irritants. TRPM8 is predominantly known for its involvement in sensing cold temperatures and is often referred to as the "cold and menthol receptor. Dysregulation of these TRP channels may result in aberrant sensory signalling, leading to the development of neuropathic pain.

Based on the time course study of behaviour responsiveness assay we observed a difference in pain intensity and duration in both models of CINP. On day 35 post the last injection of PTX the pain had returned to that baseline threshold, however, combined chemotherapy-induced pain lasted up to day 56 followed by the last injection. We determined whether these behavioural alterations are correlated with molecular studies or not. TRPs channels such as TRPA1, TRPV1 and TRPM8 play an important role in the signal transduction of peripheral to central or vice versa in CINP condition. Using one-way ANOVA followed by Tukey’s multiple comparison tests we found a

significant effect across the groups on mRNA levels of TRPA1, TRPV1 and TRPM8 in the DRG [$F_{2,9}=34.7$; $p<0.001$ and $F_{2,12}=35.3$; $p<0.001$, $F_{5,12}=35.6$; $p<0.001$ respectively] and spinal cord [$F_{2,18}=30.7$; $p<0.05$, $F_{5,12}=14.1$; $p<0.001$ and $F_{2,14}=36.3$; $p<0.001$ respectively] tissues of vehicle and chemotherapy administered rats. We found that combined chemotherapy administration has significantly increased the TRPA1, TRPV1 and TRPM8 mRNA expression in the DRG and spinal cord tissues as compared to both PTX and vehicle-treated groups **(Figure 4.4 A1-A3 & B1-B3)**. However, we did not find any significant changes in the expression of TRPA1, TRPV1 and TRPM8 mRNA expression in the PTX-treated rats as compared to the vehicle-administered group.

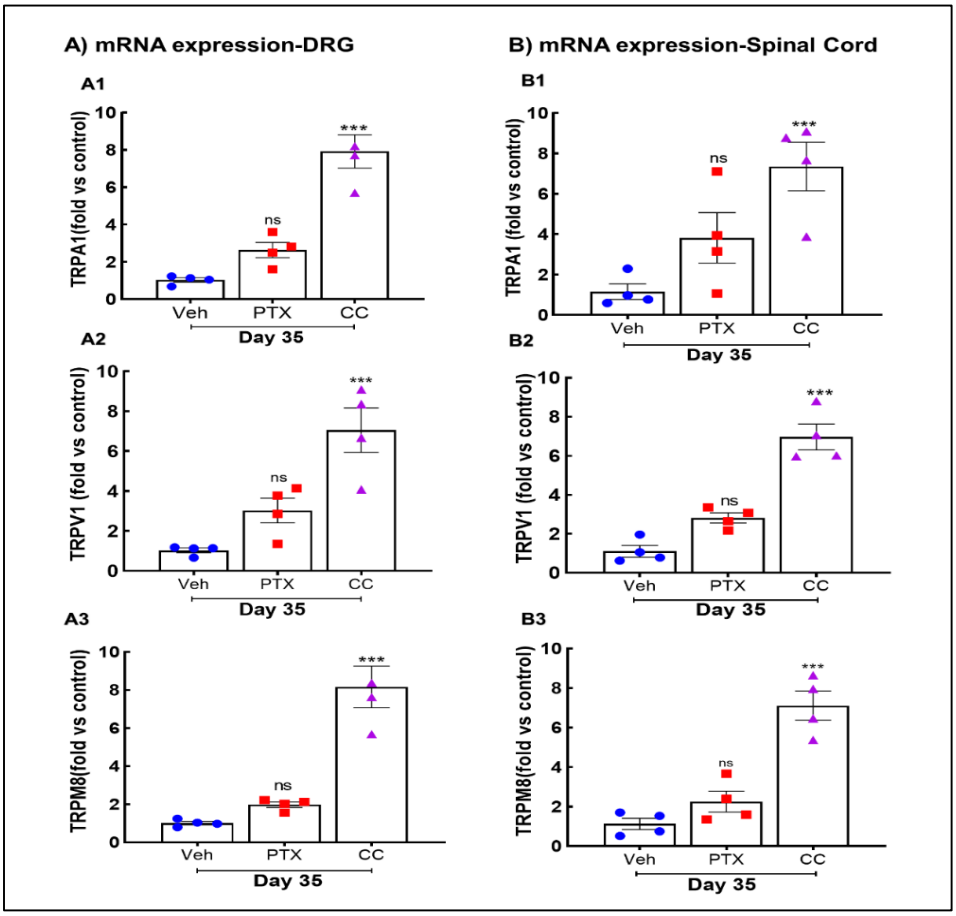

Figure 4.4. Combined chemotherapy increases TRP (TRPA1, TPPV1 and TRPM8) mRNA expression in dorsal root ganglion (DRG) and spinal cord of rats. CC-treated but not the PTX-treated rats showed significant upregulation of the TRPA1,

TPPV1 and TRPM8 mRNA levels in both DRG and spinal cord tissues of rats on day 35 post last chemo injection. **(A1-A3)** TRPA1, TRPV1 and TRPM8 mRNA expression in DRG. **(B1-B3)** TRPA1, TRPV1 and TRPM8 mRNA expression in the spinal cord. Data were presented as mean ± SEM. ***$p<0.001$ indicates statistical significance as compared to the vehicle and PTX treated rats. $p<0.05$ was considered statistically significant. n=4 biological and n=3 technical replicates. CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.3.2 Combined chemotherapy significantly upregulated NR2B mRNA expression in DRG and spinal tissue

TRP channels are prominently expressed in sensory nerve endings and play a direct role in transmitting nociceptive (pain) signals. Activation of TRP channels initiates an influx of calcium ions and triggers the release of neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP). These neuropeptides intensify pain signalling and promote neuroinflammatory responses. Moreover, the release of neuropeptides and the activation of TRP channels have downstream effects on glial cell activation, particularly microglia and astrocytes (Carozzi et al., 2015; Liedtke and Kim, 2005). This process contributes to central sensitization, a phenomenon where the central nervous system (CNS) becomes hypersensitive to pain signals, resulting in an elevated perception of pain. Additionally, neuropeptides and the inflammatory cascade can further stimulate glial cells and modulate the activity of NMDA receptors, with a specific focus on the NR2B subunit. In the context of rats treated with oxaliplatin, mechanical allodynia has been observed alongside increased expression levels of N-methyl-D-aspartate (NMDA) receptor subtype 2B (NR2B) in the spinal cord. Furthermore, downstream targets of NR2B, including nitric oxide synthase (NOS) activity and the phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), are upregulated. These molecular changes collectively contribute to the persistence and exacerbation of chronic pain states associated with CINP (Tsai et al.,

2022; Uhelski et al., 2021). Utilizing RT-PCR analysis, we examined the effect of combined chemotherapy on NR2B mRNA expression in both the dorsal root ganglia (DRG) and spinal cord of rats with CINP at day 35 post-treatment. Our findings revealed a substantial increase in NR2B mRNA expression in both the DRG ($p<0.001$) and spinal cord ($p<0.001$) of rats subjected to combined chemotherapy compared to both vehicle-treated and PTX-treated rats **(Figure 4.5 A & B).** However, there was no significant alteration in NR2B mRNA expression in the DRG and spinal cord of rats administered with PTX on day 35 following the last chemotherapy injection, when pain levels had returned to the original baseline, as compared to the vehicle-treated group Statistical analysis using one-way ANOVA followed by Tukey's multiple comparison tests demonstrated a significant effect across the groups on mRNA expression of NR2B in DRG [$F_{5,18}=34.7$; $p<0.001$] and spinal cord [$F_{5,12}=36.7$; $p<0.001$] tissues of both vehicle-treated and chemotherapy-treated rats.

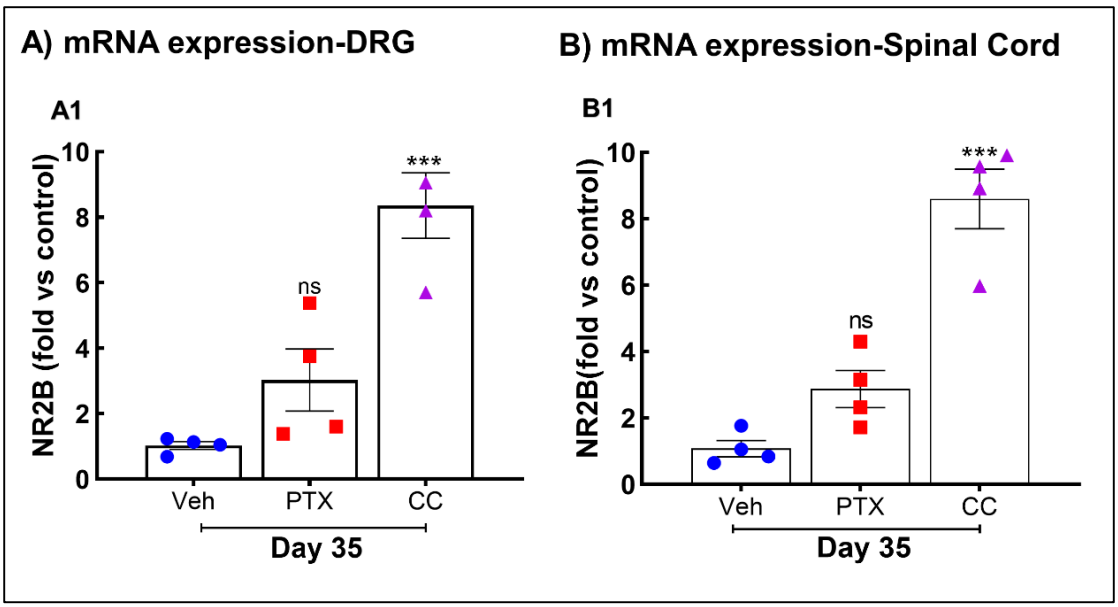

Figure 4.5 Combined chemotherapy increases NR2B mRNA expression in dorsal root ganglion (DRG) and spinal cord of rats. CC-treated but not the PTX-treated rats showed significant upregulation of NR2B mRNA levels in both DRG and spinal cord tissues of rats on day 35 post last chemo injection. **(A1)** NR2B mRNA expression in

DRG. **(B1)** NR2B mRNA expression in the spinal cord. Data were presented as mean ± SEM. ***p<0.001 indicates statistical significance as compared to the vehicle and PTX treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates. CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.3.3 Combined chemotherapy increases neuropeptides levels in the DRG and spinal cord of rats

Combined chemotherapy has been demonstrated to result in a notable increase in the mRNA levels of neuropeptides, specifically Substance P and calcitonin gene-related peptide (CGRP), within both the dorsal root ganglia (DRG) and spinal cord. Substance P and CGRP are recognized neuropeptides renowned for their pivotal roles in pain signalling and modulation. Substance P, in particular, acts as a neurotransmitter critically involved in nociceptive transmission, while CGRP is known for its potent vasodilatory properties and its implication in pain processing. Emerging evidence from various studies has consistently indicated alterations in neuropeptide expression patterns in the context of chronic pain conditions, including CINP. The surge in neuropeptide release and upregulation, as observed with Substance P and CGRP, is closely associated with heightened pain sensitivity and the initiation of neuro-inflammatory responses.

In our investigation, we further explored the expression of Substance P and CGRP in DRG and spinal tissues in CINP rats at day 35 post-treatment. Our findings unveiled a significant elevation in the mRNA expression of both Substance P and CGRP in the DRG ($p<0.001$ and $p<0.01$, respectively) and spinal cord ($p<0.001$ and $p<0.01$, respectively) tissues when compared to both the PTX-treated and vehicle-treated groups. Notably, on day 35, PTX-treated rats exhibited no substantial alterations in the expression of either neuropeptide when compared to the vehicle-treated group

(Figure 4.6 A1-A2 & B1-B2). Statistical analysis employing one-way ANOVA followed by Tukey's multiple comparison tests disclosed a significant impact across the groups regarding Substance P and CGRP mRNA expression in both DRG [$F_{2,18}=36.7$; $p<0.001$ and $F_{2,12}=35.3$; $p<0.001$, respectively] and spinal cord [$F_{2,16}=30.7$; $p<0.05$ and $F_{3,12}=34.8$; $p<0.001$, respectively] tissues of both vehicle-treated neuropathic rats.

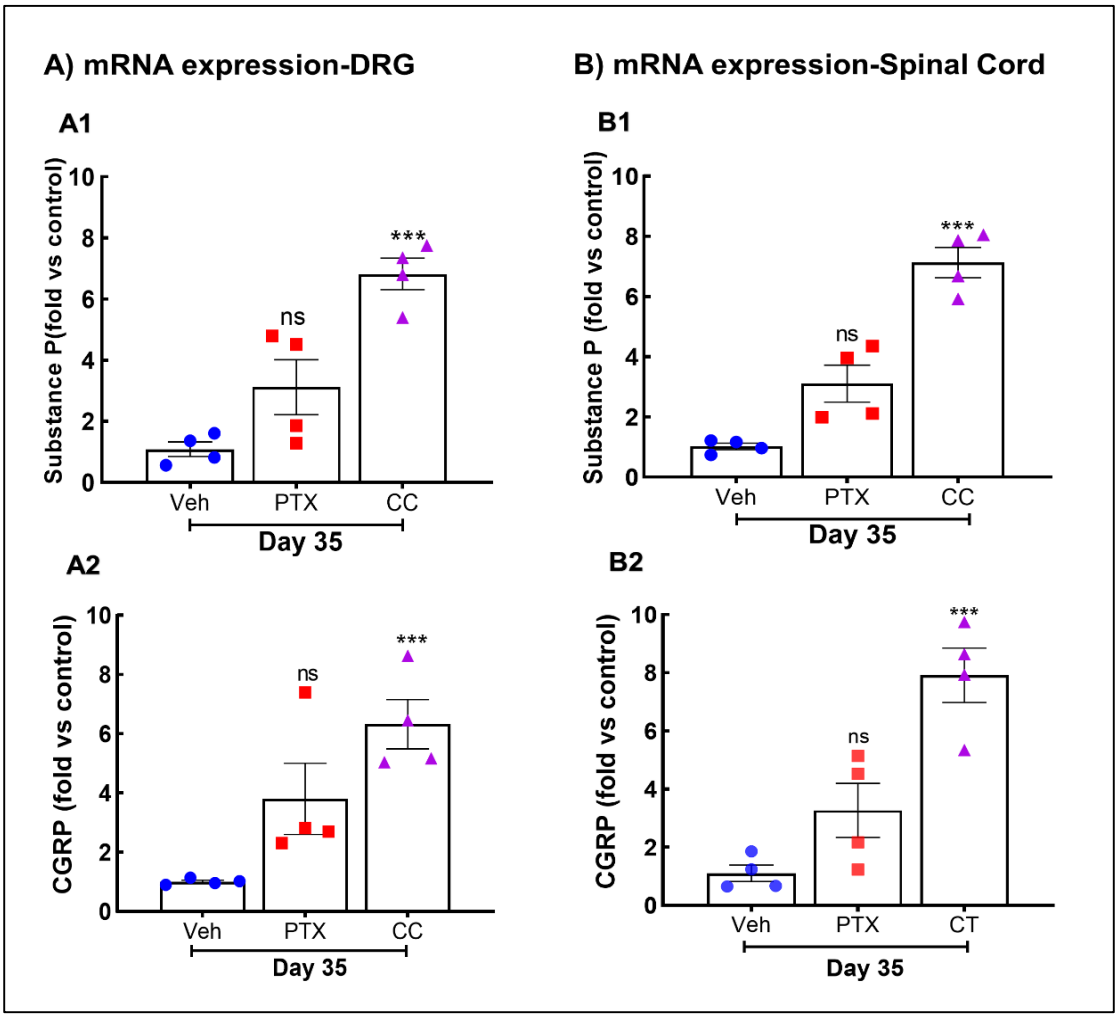

Figure 4.6 Combined chemotherapy upregulates neuropeptides levels in rats. CC-treated rats showed a significant upregulation of the mRNA expression of substance P, CGRP levels in both the DRGs and spinal tissues but PTX-treated rats do not show any significant upregulation of mRNA level of the above-mentioned makers, neuropeptide at day 35 in both DRGs and spinal level. **(A1-A2)** substance P, CGRP mRNA expression in DRG. **(B1-B2)** substance P and CGRP mRNA expression in the spinal cord. Data were

presented as mean ± SEM. ***p<0.001 indicates statistical significance as compared to the vehicle and PTX treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates. CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.3.4 Combined chemotherapy-induced neuro-inflammation in DRG and spinal cord of rats

Pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), play a pivotal role in the pathophysiology of painful neuropathy. They exert their influence by directly activating nociceptors or via indirect pathways such as oxidative stress. Our research outcomes have unveiled that combined chemotherapy treated rats exhibited significantly elevated levels of TNF-α and IL-1β at day 35 as compared to both the vehicle-treated and PTX-treated groups. Using one-way ANOVA followed by Tukey's multiple comparison test suggested significant effect across the groups TNF-α and IL-1β mRNA expression in L4-L5 and L6 dorsal root ganglia (DRG) [$F_{2,38}=42.7$; $p<0.001$ and $F_{2,14}=35.3$; $p<0.001$, respectively] as well as spinal cord [$F_{2,18}=30.7$; $p<0.05$ and $F_{2,12}=14.1$; $p<0.001$, respectively] tissues of rats treated with vehicle, PTX, and combined chemotherapy. Intriguingly, PTX treatment also led to a significant increase in the expression of TNF-α at day 35 compared to the vehicle-treated group. However, it is worth noting that the expression of IL-1β in the PTX group did not exhibit a significant difference compared to the vehicle-treated group **(Figure 4.7 A1-A2 and B1-B2).**

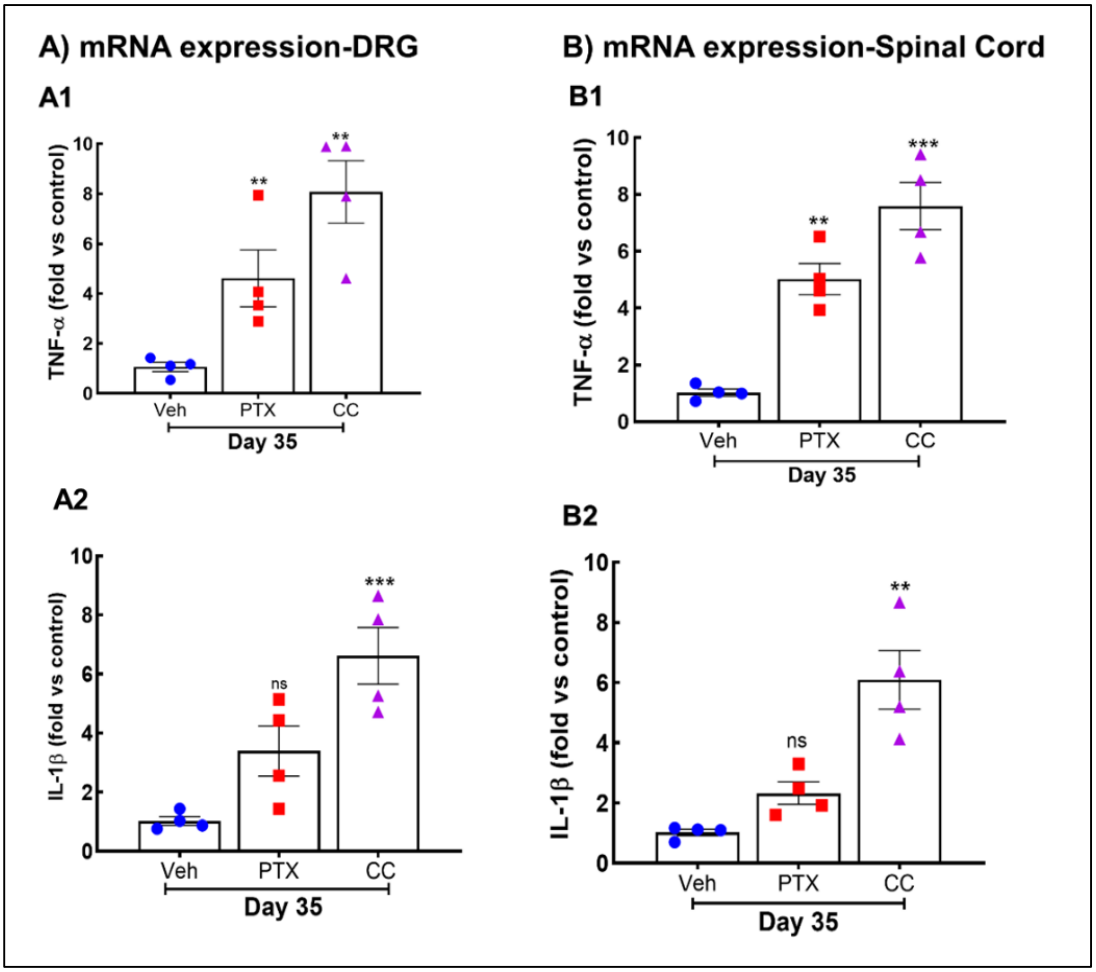

Figure 4.7 Combined chemotherapy-induced neuro-inflammatory markers in rats. CC-treated rats showed a significant upregulation of the mRNA expression of TNF-α and IL-1β levels in both the DRGs and spinal tissues but PTX-treated rats do not show any significant upregulation of mRNA level of the above-mentioned makers, inflammatory mediators at day 35 in both DRGs and spinal level. **(A1-A2)** TNF-α and IL-1β mRNA expression in DRG. **(B1-B2)** TNF-α and IL-1β mRNA expression in the spinal cord. Data were presented as mean ± SEM. ***p<0.001and **p<0.01 indicates statistical significance as compared to the vehicle and PTX-treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates. CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.4 Biochemical observations and interpretation

4.3.4.1 Combined chemotherapy suppressed the antioxidant enzyme activity in sciatic nerve of rats

While the neurotoxicity induced by different classes of chemotherapeutic drugs may manifest differently, it invariably converges on a common outcome: peripheral

neuronal degeneration or small fiber neuropathy, hallmark features of CINP. Oxidative stress has emerged as a central player contributing to neuronal damage across various neuropathy models, encompassing diabetic neuropathy and CINP. It is increasingly recognized that chemotherapy-induced mitochondrial dysfunction, accompanied by the generation of oxidative stress, constitutes a pivotal factor in the detriment to peripheral nerves. Empirical evidence robustly supports the involvement of mitochondria-mediated oxidative and nitrosative stress in the genesis of peripheral nerve damage. The elucidation of these intricate mechanisms holds promise for the identification of novel biomarkers to enhance our understanding of CIPN and to advance therapeutic interventions. Therefore, the assessment of markers such as malondialdehyde, glutathione (GSH), and superoxide dismutase (SOD) represents a valuable approach for monitoring the progression of peripheral neuropathy and gauging the response to treatment. In our study, combined chemotherapy treated rats displayed a substantial ($p<0.001$) reduction in the enzymatic level of GSH within the sciatic nerve, as compared to both the vehicle-treated and PTX-treated groups, at day 35 following the administration of the last chemotherapy dose. Statistical analysis, employing one-way ANOVA followed by Tukey's multiple comparison test, unveiled a significant effect across the groups concerning GSH level in the sciatic nerve [$F_{4,30}=125.57$; $p<0.001$] (**Figure 4.8 A**). A similar pattern emerged in the levels of the antioxidant enzyme SOD within the sciatic nerve of rats administered combined chemotherapy (**Figure 4.8 B**). Following one-way ANOVA followed by Tukey's multiple comparison test suggested a significant effect across the groups in terms of SOD activity within the sciatic nerve, compared to both the vehicle-treated and PTX-treated rats [$F_{5,24}=125.57$; $p<0.001$].

Intriguingly, we did not observe any alterations in the levels of antioxidant enzymes in PTX-treated rats compared to the vehicle group on day 35.

4.3.4.2 Combined chemotherapy increases MDA and Nitrite levels in sciatic nerve of rats

Chemotherapy has been consistently associated with an elevation in the levels of malondialdehyde (MDA) and nitrite within the sciatic nerve of rats experiencing chemotherapy-induced peripheral neuropathy (CINP). These biomarkers hold significance as they are indicative of critical processes contributing to the pathogenesis of CINP. MDA serves as a reliable marker of lipid peroxidation and oxidative stress, reflecting the imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defence mechanisms. MDA levels within the sciatic nerve of rats subjected to combined chemotherapy, when compared to both vehicle and PTX-treated rats at day 35 post the last chemotherapy injection. The significant effect across the groups regarding MDA level in the sciatic nerve is apparent following one-way ANOVA followed by Tukey's multiple comparison test [$F_{5,26}=65.24$; $p<0.001$]. Intriguingly, PTX treatment does not exhibit any significant impact on either MDA or nitrite levels at day 35 when compared to vehicle-treated rats (**Figure 4.8C**). These findings highlight the robust correlation between chemotherapy-induced oxidative and nitrosative stress and the development of peripheral nerve damage in the context of CINP. Similarly, an analogous pattern emerges in the case of nitric oxide that may react with superoxide radicals, forming peroxynitrite, a highly reactive and destructive species that further amplifies oxidative stress and inflammation within the CINP context. Notably, combined chemotherapy treatment significantly amplifies nitrite levels within the sciatic nerve of neuropathic rats at day 35 following the last

chemotherapy injection. Statistical analysis, utilizing one-way ANOVA followed by Tukey's multiple comparison test, emphasizes the substantial effect across the groups concerning nitrite activity in the sciatic nerve [(F5,24=56.24; p<0.001), p<0.001, n=4] (**Figure 4.8D).**

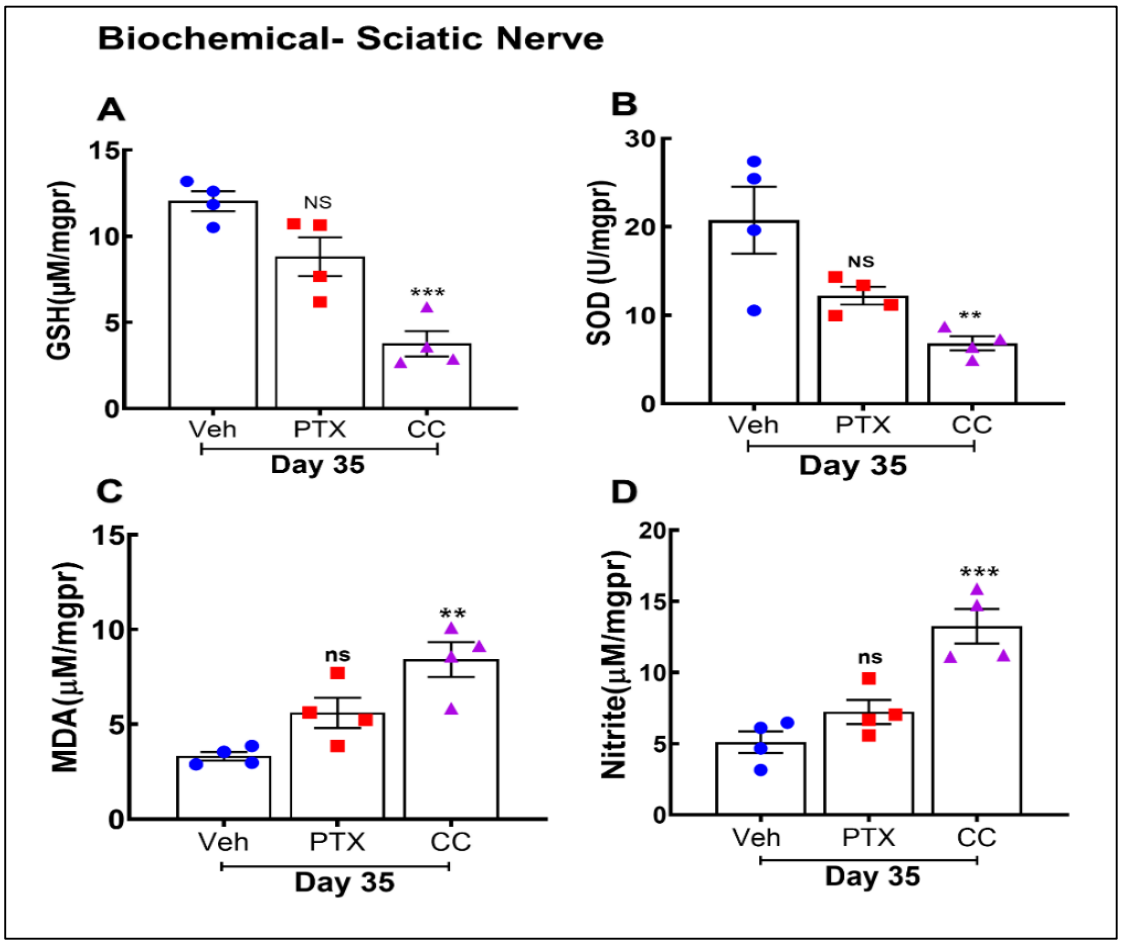

Figure 4.8. Combined chemotherapy causes biochemical changes in rats. Biochemical alterations: CC-treated rats showed significantly decreased GSH and SOD levels and increased level of MDA and nitrite in the sciatic nerve of rats **(A-D)**. Data were presented as mean ± SEM. ***p<0.001and **p<0.01 indicates statistical significance as compared to the vehicle and PTX treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates. CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.5 Pharmacological validation of novel combined chemotherapy model of CINP

To assessed the pharmacological validity of our novel combined chemotherapy model of CINP, we introduced gabapentin treatment in separate rat cohorts. This approach aimed to assess the predictive validity of our model in terms of its ability to mimic the response to therapeutic interventions. Notably, our observations revealed that the repetitive administration of gabapentin did not yield any significant effect on the reduction in body weight induced by either the combined chemotherapy regimen or PTX chemotherapy alone in the rat cohorts. Subsequently, we conducted a comprehensive series of behavioural and molecular analyses to delve into the effects of standard drug treatment in neuropathic rats. This investigation allowed us to assess the efficacy of gabapentin and gain insights into its potential in mitigating the neuropathic symptoms associated with our established CINP model.

4.3.5.1 Gabapentin does not affect body weight in chemotherapy treated rats

We investigated the influence of gabapentin on daily body weight fluctuations in rats with neuropathic conditions. Using Two-Way ANOVA followed by Bonferroni's multiple comparison tests indicated a noteworthy ($p < 0.001$) reduction in the body weight of rats subjected to combination chemotherapy, as compared to their respective baseline values. In contrast, Gabapentin exhibited no significant impact on body weight changes in rats subjected to chemotherapy when compared to their baseline measurements **(Figure 4.9)**.

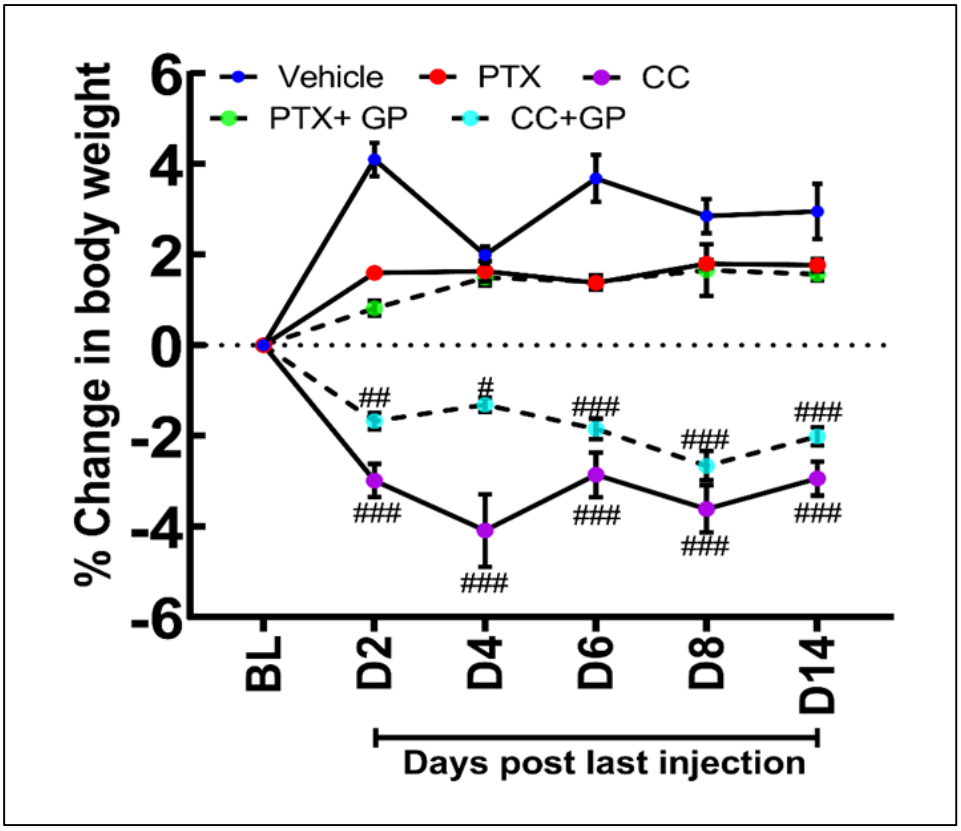

Figure 4.9 Effect of gabapentin on daily body weight changes in neuropathic rats. Treatment with CC agents significantly decreased the body weight of rats. Gabapentin did not show any significant effect on body weight changes in chemotherapy-treated rats. Data were present in Mean ± SEM. #p<0.05, ##p<0.01 and ###p<0.001 indicates. p<0.05 was considered statistically significant. n=7-8. CC: Combination chemotherapy, PTX: Paclitaxel, GP: Gabapentin.

4.3.5.2 Gabapentin alleviates chemotherapy-induced mechanical and thermal pain behaviour in rats

To evaluate the effect of gabapentin on pain sensitivity in both the combined chemotherapy and PTX models of CINP, the drug was administered at a dose of (60 mg/kg i.p.) daily, commencing from day 1 and continuing for 8 days concurrently with the chemotherapy treatment. This repetitive intraperitoneal administration of gabapentin effectively prevented the development of chemotherapy-induced thermal hyperalgesia in both CINP models (p<0.001) (**Figure 4.10 A-B**).

Combination chemotherapy and PTX treated rats resulted in a significant reduction in the paw withdrawal threshold in neuropathic rats, demonstrating a time-dependent decrease in pain threshold. However, this chemotherapy-induced hyperalgesia was notably attenuated by the repetitive administration of gabapentin (60 mg/kg i.p.). Repeated-measures two-way ANOVA analysis revealed a substantial effect both across the different treatment groups [F5, 46=174; $p<0.001$] and at various time points [F_{4}.75,199=155; $p<0.001$] in terms of paw withdrawal thresholds and paw withdrawal latency [$F_{5, 46}$=174, $p<0.001$] and time points [$F_{4.55,}$ =155, $p<0.001$] among the vehicle-treated, PTX-treated, and combined chemotherapy-treated rats (**Figure 4.10 C-D**). These results underscore the therapeutic potential of gabapentin in mitigating the pain associated with CINP in a dose-dependent manner.

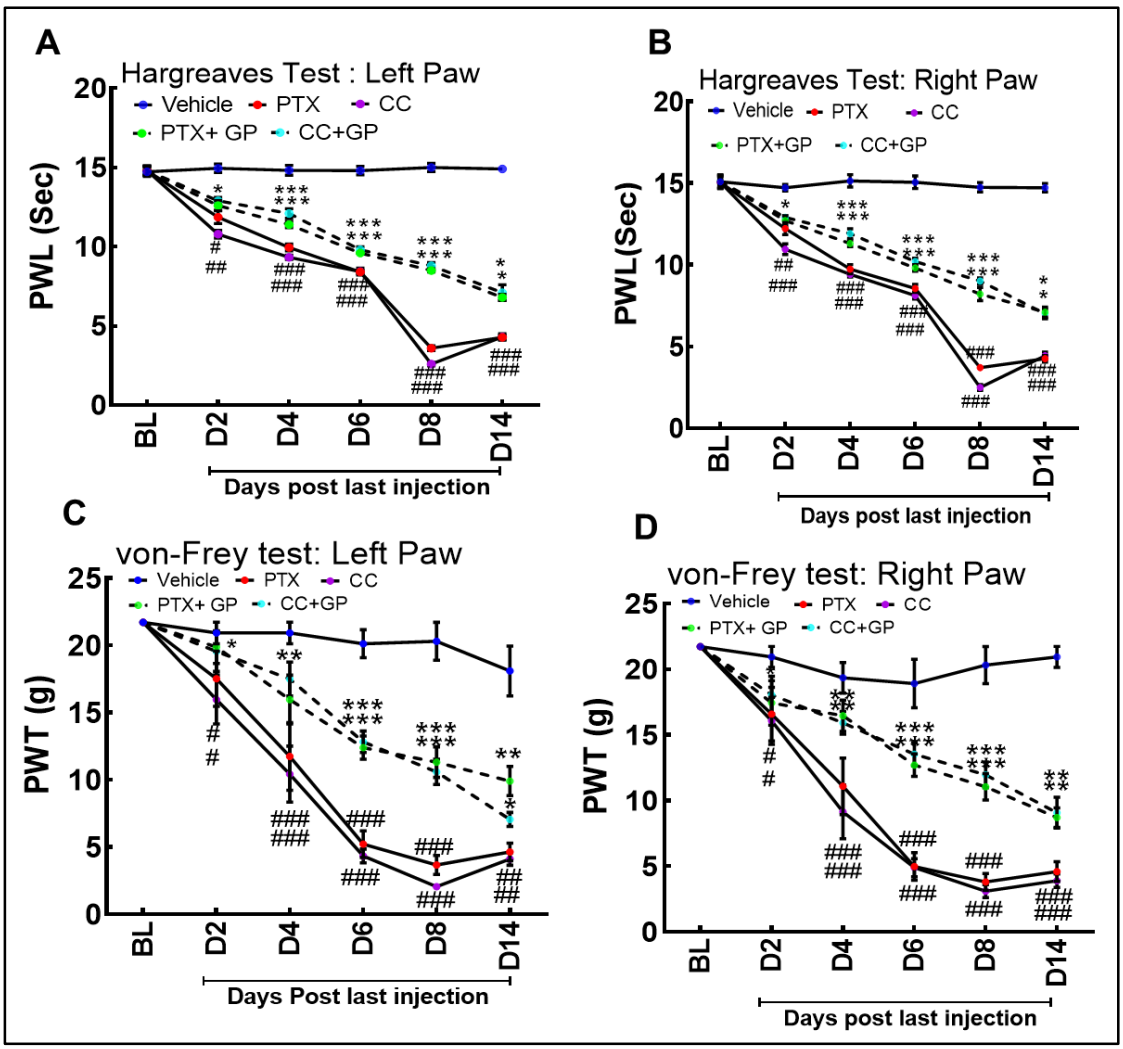

Figure 4.10 Effect of gabapentin on thermal and mechanical pain behavior in neuropathic rats. Both CC and PTX caused significantly decreased thermal hyperalgesia, mechanical allodynia of rats on day 14 post last injection of chemotherapy as compared to their baseline. GP administration (60 mg/kg i.p.) attenuates the development of pain due to chemotherapy in both CC and PTX alone-treated rats as compared to their pre-drug baseline and vehicle treated neuropathic rats. **Hargreaves test (A)** paw withdrawal latency in left hind paw **(B)** paw withdrawal latency in right hind paw. **von Frey hair test (C)** paw withdrawal threshold in left hind paw **(D)** paw withdrawal threshold in the right hind paw. Data were presented as mean ± SEM. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ indicates statistical significance as compared to the relative baseline and vehicle-treated rats and. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the rat received only PTX and CC. p<0.05 was considered statistically significant. PWT: Paw withdrawal threshold, PWL: Paw withdrawal latency, CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.5.3 Gabapentin alleviates chemotherapy-induced cold pain behaviour in rats

Gabapentin emerges as a potent alleviator of chemotherapy-induced cold pain behaviours in rats. In our experimental paradigm, the combined chemotherapy and PTX treatment induced a significant increase in cold-evoked responses, characterized by behaviours such as paw lifts and prolonged licking duration in the rats, as compared to their baseline and vehicle-treated control rats. However, the chronic administration of gabapentin proved highly effective in attenuating the development of cold-induced pain resulting from chemotherapy in both CINP models. Employing repeated-measures two-way ANOVA, we demonstrated a substantial effect, both across the different treatment groups [$F_{5,42}=173$; $p<0.001$] and at various time points [$F_{4.89,199}=185$; $p<0.001$], in the response scores of the rats subjected to vehicle, PTX, and combined chemotherapy treatments (**Figure 4.11 A-B**).

Moreover, our assessment using the acetone evaporation test revealed that both PTX-treated and combined chemotherapy-treated rats exhibited a significant increase in paw withdrawal responses, indicative of heightened cold sensitivity. Remarkably, these heightened cold pain responses were significantly attenuated by the administration of gabapentin (60 mg/kg i.p.). (**Figure 4.11 C-D**). The results from repeated-measures two-way ANOVA, followed by Bonferroni's multiple comparison tests, underscore the significant effect observed across the groups [$F_{5,48}=174$; $p<0.001$] and time points [$F_{4.89,199}=185$; $p<0.001$] in terms of response scores among the vehicle-treated, PTX-treated, and combined chemotherapy-treated rats. These compelling findings collectively indicate that gabapentin effectively prevents the neuropathic-like cold pain behaviours induced by combined chemotherapy and PTX

treatment in CINP rats, emphasizing its therapeutic potential in ameliorating CINP-related discomfort and hypersensitivity.

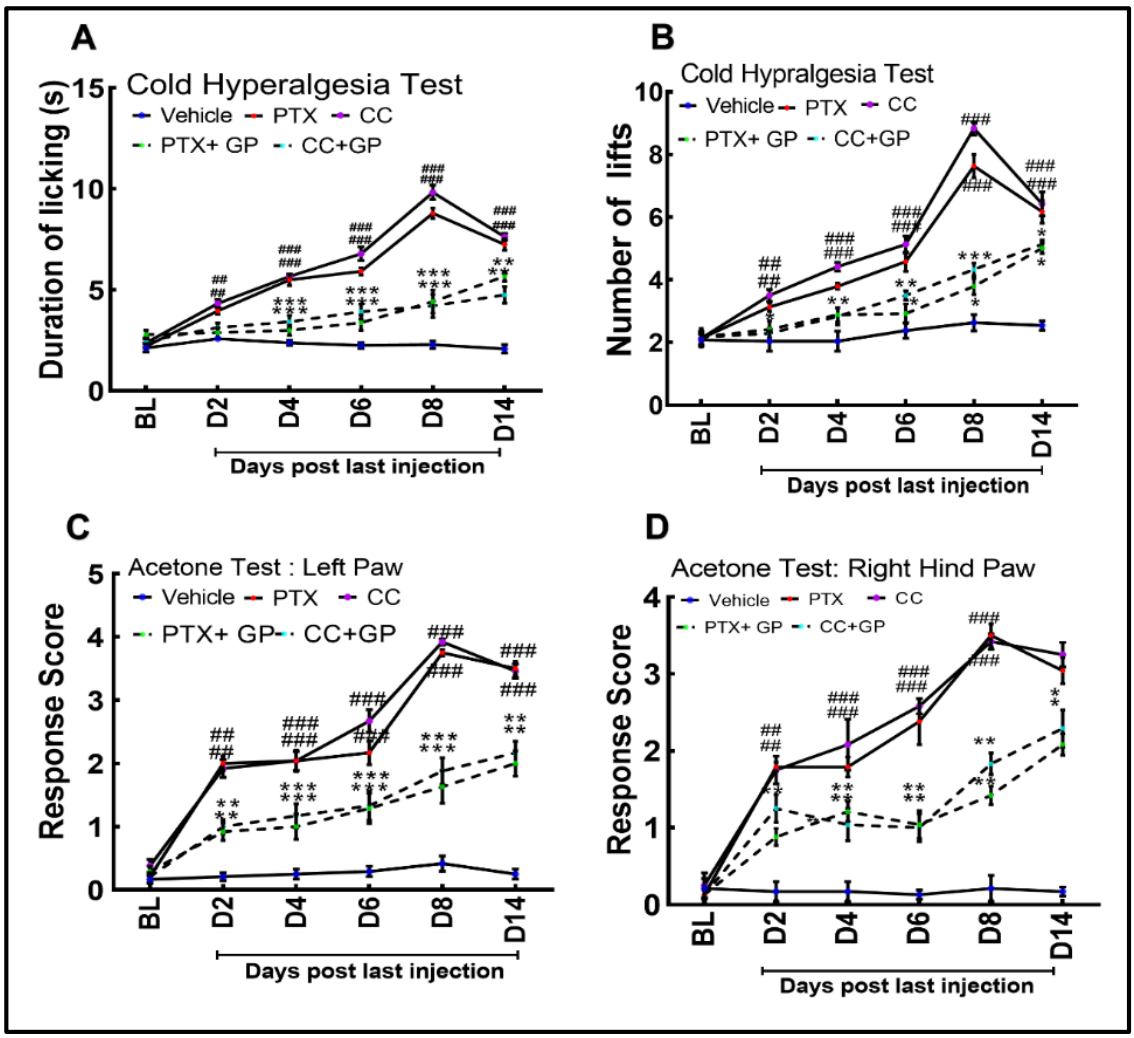

Figure 4.11 Effect of gabapentin on cold hypersensitivity of neuropathic rats. Both CC and PTX caused significantly elevated cold hyperalgesia and cold allodynia of rats on day 14 post last injection of chemotherapy as compared to their baseline. GP administration (60 mg/kg i.p.) attenuates the development of pain due to chemotherapy in both CC and PTX alone-treated rats as compared to their pre-drug baseline and vehicle treated neuropathic rats. **Cold hyperalgesia test (A)** duration of licking **(B)** a number of lifts/licks. **Acetone test (C)** Response Score in left hind paws **(D)** Response Score in right hind paws. Data were presented as mean ± SEM. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ indicates statistical significance as compared to the relative baseline and vehicle-treated rats and. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the rat received only PTX and CC. p<0.05 was considered statistically significant. PWT: Paw withdrawal threshold, PWL: Paw withdrawal latency, CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.5.4 Gabapentin suppressed mRNA expression of TRPV1, TRPA1 and TRPM8 in DRG and spinal cord tissue of CINP rats

TRP channels play a pivotal role in the development and progression of chronic pain conditions induced by chemotherapy. In our study, we investigated the effect of repetitive gabapentin administration on the mRNA expression levels of specific TRP channels in combined chemotherapy treated rats and paclitaxel (PTX) regimen, aiming to shed light on potential mechanisms underlying gabapentin's analgesic effects. Our findings revealed that the repetitive administration of gabapentin led to a significant reduction in the mRNA expression levels of three key TRP channels: TRPA1 ($p<0.001$), TRPV1 ($p<0.001$), and TRPM8 ($p<0.001$) in both the DRG and spinal cord of rats exposed to the combined chemotherapy and PTX treatment as compared to vehicle-treated rats (**Figure 4.12 A1-A3 & BA1-B3**). Remarkably, the observed results for TRPM8 were somewhat surprising. Unlike TRPA1 and TRPV1, there were no significant changes in TRPM8 mRNA expression levels in both the DRG and spinal cord of PTX-treated rats as compared to vehicle-treated group. Furthermore, gabapentin did not exert any noticeable effect on the expression levels of TRPM8 in these tissues (**Figure 4.12 A3&B3**). These outcomes suggest that gabapentin's analgesic effects in our novel combined chemotherapy model may involve mechanisms that specifically target TRPV1, TRPA1, and TRPM8-dependent pathways. This aligns with previous reports on the role of gabapentin in alleviating chemotherapy-induced neuropathic pain (CINP) through the modulation of TRP channels (Jain et al., 2023). By influencing the expression and function of these TRP channels, gabapentin likely contributes to the attenuation of sensory hypersensitivity and pain associated with CINP, offering promising insights for the development of targeted therapeutic strategies.

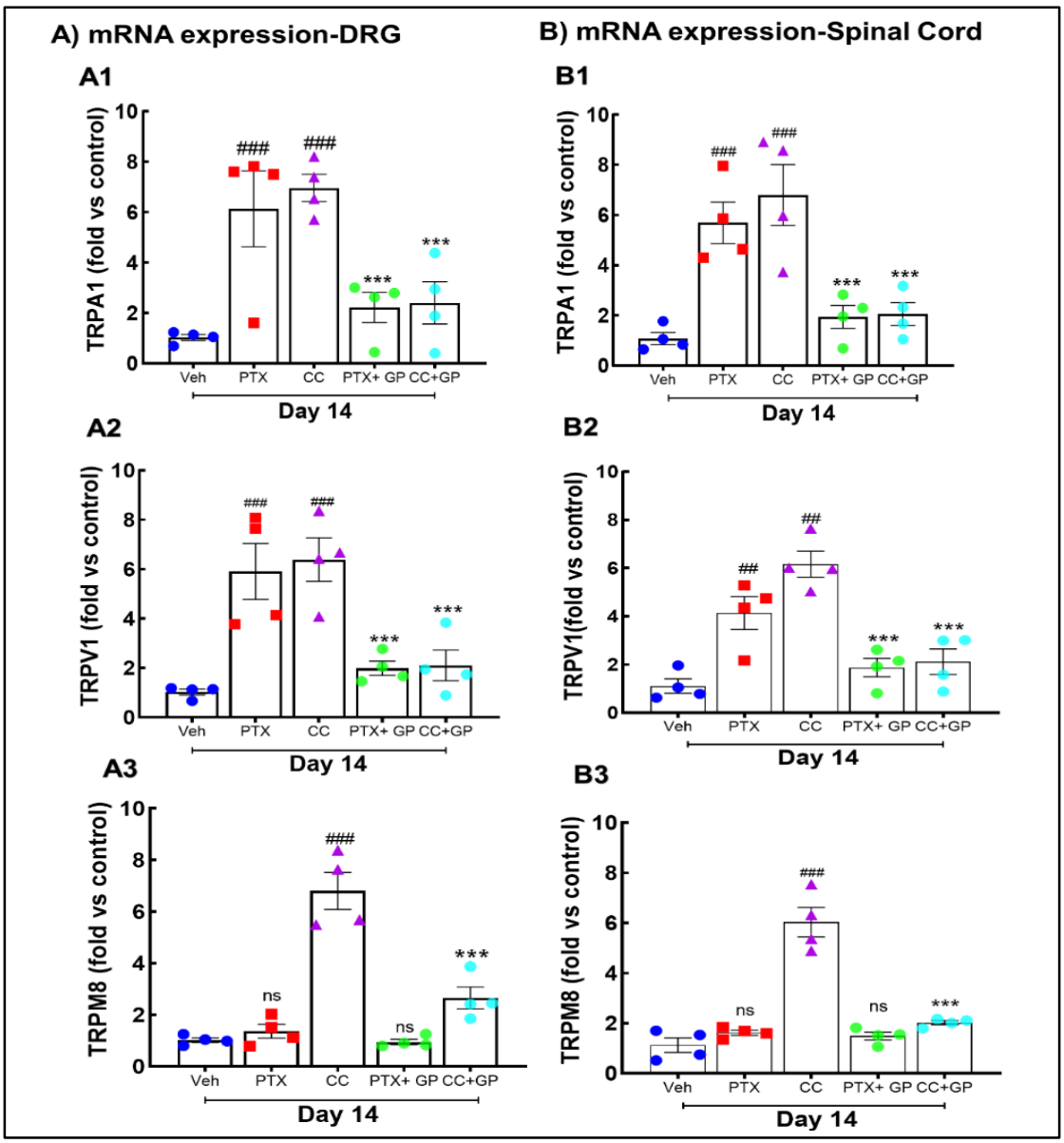

Figure 4.12. Effect of gabapentin on chemotherapy induced-TRP (TRPA1, TPPV1, TRPM8) channel mRNA expression in dorsal root ganglion (DRG) and spinal cord of rats. CC-induced increased mRNA expressions of TRPA1, TRPV1, and TRPM8 which were significantly reversed on treatment with GP (60mg/kg i.p.) on day 14. However, TRPM8 expression was unaltered in PTX-treated rats and gabapentin does not have any effect on TRPM8 expression in the PTX-treated rats. **(A1-A3)** TRPA1, TRPV1, TRPM8 mRNA expression in DRG. **(B1-B3)** TRPA1, TRPV1, TRPM8 mRNA expression in the spinal cord. Data were presented as mean ± SEM. ###p<0.001and ##p<0.01 indicates statistical significance as compared to the vehicle-treated rats. ***p<0.001 indicates statistical significance as compared to the CC and PTX-treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates. CC: Combined chemotherapy, PTX: Paclitaxel.

4.3.5.5 Gabapentin treatment decreases NR2B mediated Iba1 expressions in DRG and spinal cord of neuropathic rats

Gabapentin treatment has been observed to significantly reduce the expression of NR2B in DRG and spinal tissues of rats afflicted with CINP. Neuro-inflammation, which arises in response to tissue injury or immune cell activation, plays a pivotal role in enhancing the transmission of pain signals through nociceptors located in the DRGs and spinal cord. In the context of CINP, the morphological and functional characteristics of glial cells undergo alterations. These changes often coincide with the activation of NR2B receptors. Taking into account the existing body of evidence, our study delved into NR2B-mediated mechanisms along with two classical markers of microglial activation Iba1 within the DRG and spinal tissue of neuropathic rats. Our analysis, employing one-way ANOVA followed by Tukey's multiple comparison test, unveiled a noteworthy increase in the protein expression of NR2B and Iba1 in both the DRGs ($p<0.01$, $p<0.001$) and ($p<0.05$, $p<0.01$) spinal cord ($p<0.05$, $p<0.01$) ($p<0.01$, $p<0.001$) of rats subjected to combined chemotherapy and paclitaxel (PTX) treatment (**Figure 4.13 A1-A2 & B1-B2)**. Crucially, the administration of gabapentin resulted in a significant reduction in the elevated NR2B and Iba1($p<0.05$, $p<0.01$) protein expression induced by combined chemotherapy and PTX in both DRG ($p<0.05$, $p<0.001$) and spinal tissues ($p<0.01$, $p<0.001$). This compelling finding underscores the pivotal role of NR2B in the pathogenesis of CINP. These results align with previous research and underscore the potential of gabapentin in modulating NR2B and Iba1 expressions, highlighting its significance as a therapeutic agent in the context of CINP.

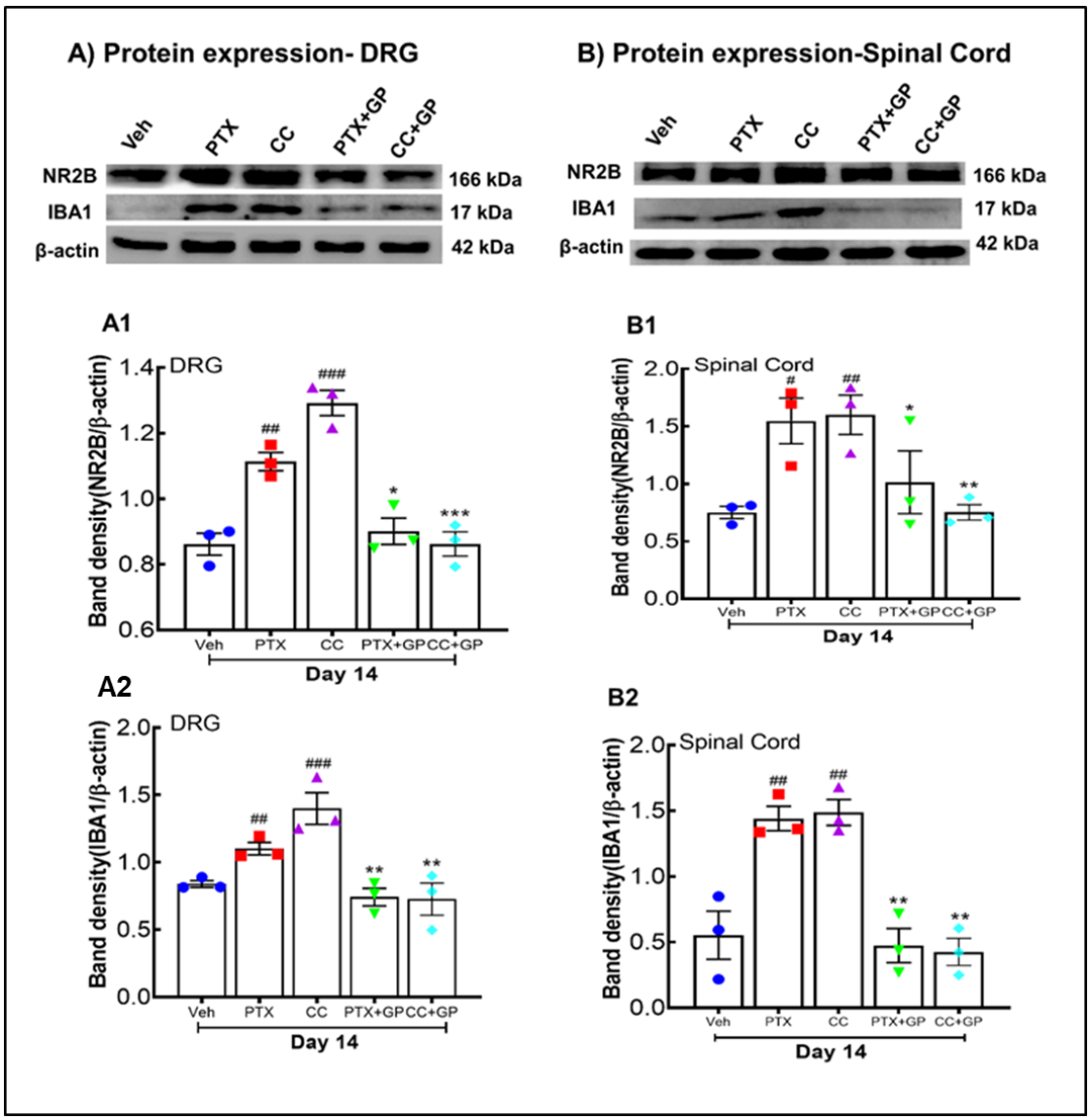

Figure 4.13 Effect of gabapentin on protein expressions of NR2B and Iba1 in dorsal root ganglion and spinal cord of neuropathic rats. **(A) DRG protein expressions (A1-A3)** GP treatment (60 mg/kg i.p.) attenuates the chemotherapy-induced increase in protein expressions of NR2B and Iba1 in DRG of neuropathic rats at day 14. **(B) Spinal protein expressions (B1-B3)** chemotherapy-induced significant increase in protein expressions of NR2B and Iba1 in the lumbar region of the spinal cord of rats which was significantly attenuated on treatment with GP 60 mg/kg i.p. at day 14. Data were presented as mean ± SEM. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ indicates statistical significance as compared to the vehicle-treated rats. *p<0.05, **p<0.01 and ***p<0.001 indicates statistical significance as compared to the rat that received only PTX and CC. p<0.05 was considered statistically significant. n=3. CC

4.3.5.6 Gabapentin treatment decreases ICAM-1 expression in DRG and spinal cord of neuropathic rats

Gabapentin treatment has been found to substantially reduce the expression of ICAM-1 in the DRG and spinal tissues of rats afflicted with CINP. Glial cells, integral to the peripheral and central surveillance systems, undergo morphological and functional alterations in CINP. Therefore, our study examined NR2B-mediated mechanisms alongside a classical markers of microglial activation ICAM-1 within the DRG and spinal tissue of CINP-affected rats. Our Western blotting results revealed a significant upregulation in the expression of ICAM-1 in both the DRGs ($p<0.001$) and spinal cord ($p<0.01$) of rats subjected to combined chemotherapy and paclitaxel (PTX) treatment (**Figure 4.14 A1 & B1**). Notably, the administration of gabapentin led to a substantial reduction in the heightened expression of ICAM-1 induced by combined chemotherapy and PTX in the DRG tissues ($p<0.05$, $p<0.001$) and spinal tissues ($p<0.01$, $p<0.001$), particularly at a dose of 60 mg/kg as compared to rats treated PTX alone. These results align with previous research and underscore the potential of gabapentin in modulating ICAM-1 expression, highlighting its significance as a therapeutic agent in the context of CINP.

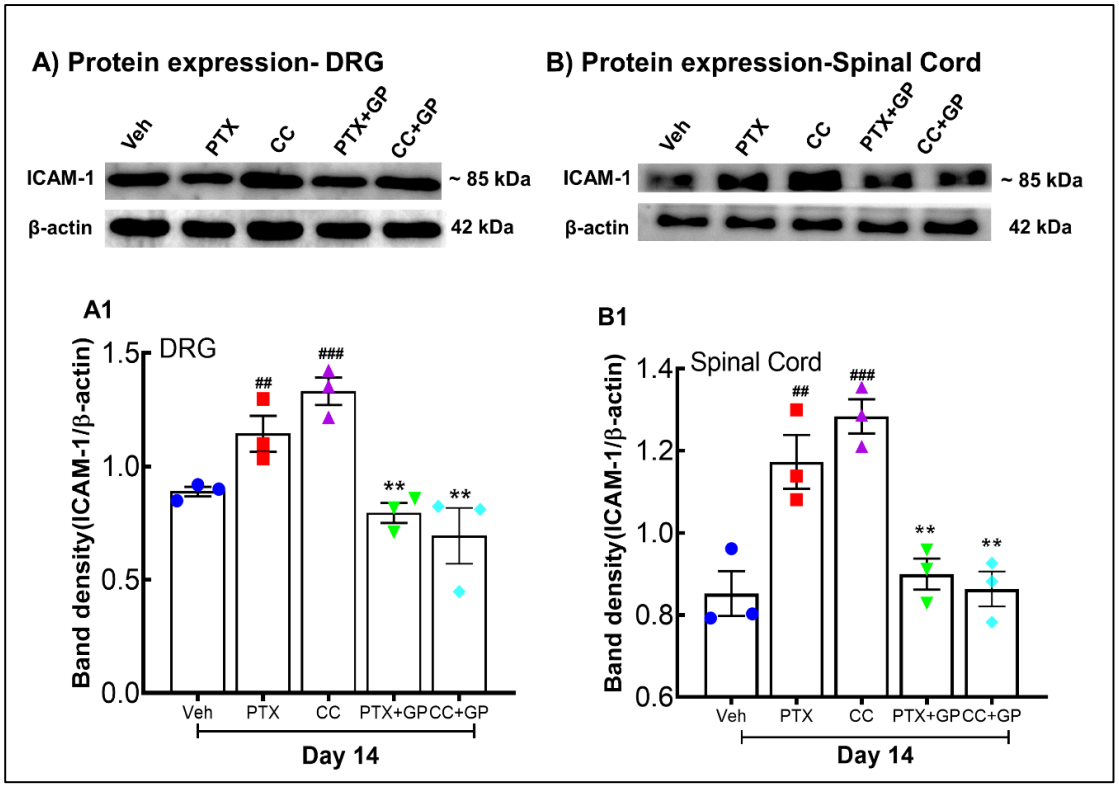

Figure 4.14 Effect of gabapentin on protein expressions of ICAM-1 in dorsal root ganglion and spinal cord of CINP rats. **(A) DRG protein expressions (A1)** GP treatment (60 mg/kg i.p.) attenuates the chemotherapy-induced increase in protein expressions of ICAM-1 in DRG of CINP rats at day 14. **(B) Spinal protein expressions (B1)** chemotherapy-induced significant increase in protein expressions of ICAM-1 in the lumbar region of the spinal cord of rats which was significantly attenuated on treatment with GP 60 mg/kg i.p. at day 14. Data were presented as mean ± SEM. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ indicates statistical significance as compared to the vehicle-treated rats. **p<0.01 indicates statistical significance as compared to the rat that received only PTX and CC. p<0.05 was considered statistically significant. n=3-4.

4.3.5.7 Gabapentin treatment suppressed CGRP and Substance P in CINP rats

The release of neuropeptides including substance P and CGRP occurs in chronic pain conditions especially associated with chemotherapy treatment. Gabapentin treatment has demonstrated its ability to suppress the expression of calcitonin gene-related peptide (CGRP) and substance P in rats affected by chemotherapy-induced peripheral neuropathy (CINP). Neuroinflammatory markers and pro-inflammatory cytokines are known to play pivotal roles in the initiation and persistence of CIPN. Both

chemotherapy models employed in this study induced an upregulation in the expression of CGRP and substance P. Remarkably, gabapentin, administered at a dose of 60 mg/kg, effectively mitigated these increases in both the DRG ($p<0.001$ and $p<0.001$, respectively) and spinal cord ($p<0.001$ and $p<0.001$, respectively) of the rats (**Figure 4.15 A1-A2 & B1-B2**). Our analysis, utilizing one-way ANOVA followed by Tukey's multiple comparison test, demonstrated a significant effect across the groups in terms of CGRP and substance P mRNA expression in both DRG [$F_{5,19}=34.7$; $p<0.001$ and $F_{5,14}=45.3$; $p<0.001$, respectively] and spinal cord [$F_{5,18}=40.7$; $p<0.05$ and $F_{5,16}= 34.1$; $p<0.001$, respectively] tissues of both vehicle and neuropathic rats. These findings align with previous research and underscore the potential of gabapentin in modulating the expression of CGRP and substance P, providing valuable insights into its therapeutic role in managing CINP. The suppression of these neuropeptides is particularly significant, as they are known to amplify pain signalling and contribute to neuroinflammatory responses, highlighting the promising pharmacological properties of gabapentin in ameliorating neuropathic pain associated with chemotherapy.

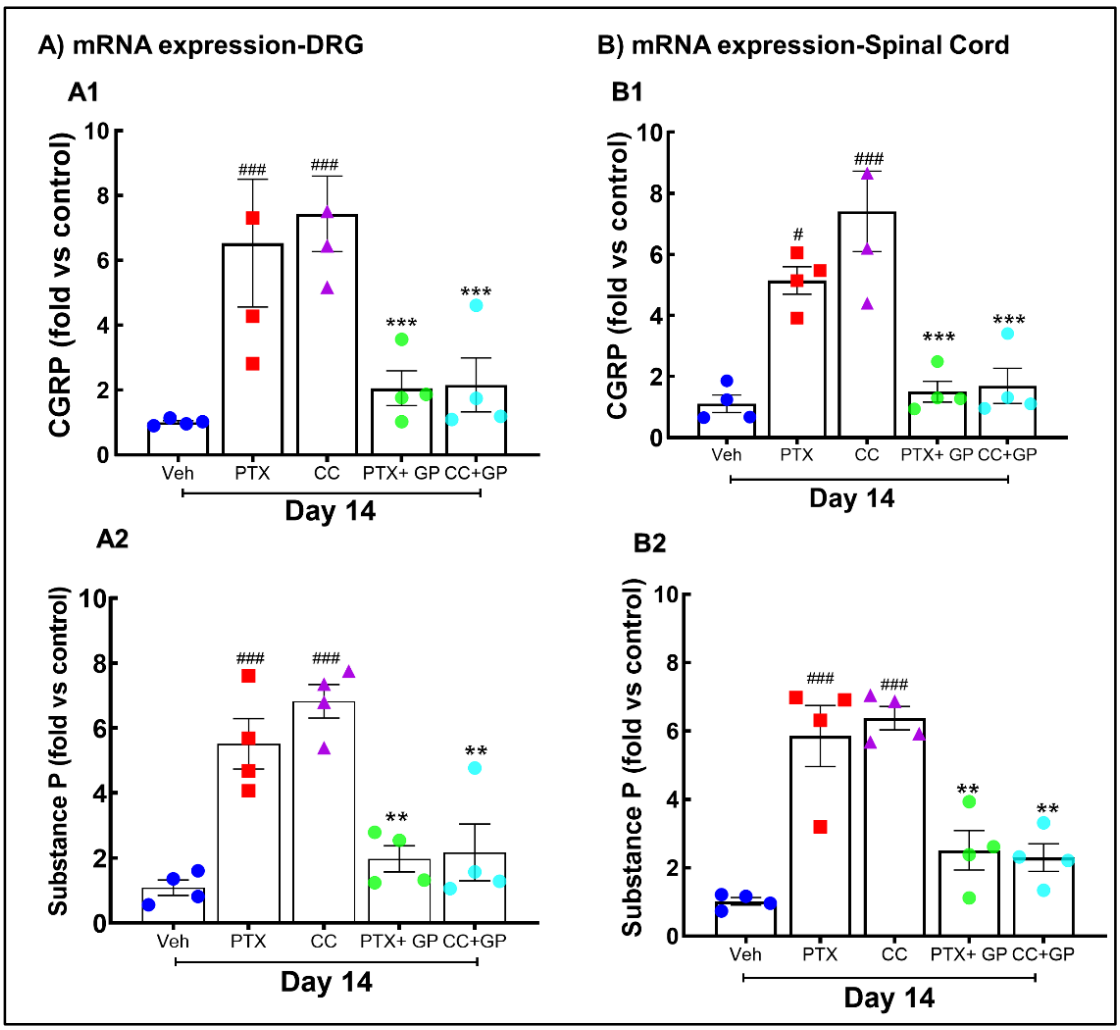

Figure 4.15 Effect of gabapentin on chemotherapy-induced upregulated neuropeptides in rats. Both CC and PTX treatment causes a significant increase in the mRNA expression of CGRP and substance P levels in both DRG and lumbar region of the spinal cord which was significantly attenuated with gabapentin administration at day 14. **(A1-A2)** CGRP and substance P mRNA expression in DRG. **(B1-B2)** CGRP and substance P mRNA substance P mRNA expression in spinal cord. Data were presented as mean ± SEM. $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ indicates statistical significance as compared to the vehicle-treated rats. ***p<0.001and **p<0.01 indicates statistical significance as compared to the vehicle and PTX-treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates.

4.3.5.8 Gabapentin treatment suppressed neuro-inflammatory cascade in neuropathic rats

Gabapentin emerges as a promising intervention in the realm of CINP, building upon established findings associated with various chemotherapeutic agents, including paclitaxel (PTX). CINP is marked by a multifaceted neuro-inflammatory cascade, characterized by heightened neuro-inflammation, augmented neuropeptide expression, and increased pro-inflammatory cytokines, all culminating in the development and persistence of neuropathic pain. Previous investigations cantered on PTX-induced neuropathy, have unveiled the pivotal role of these inflammatory processes in the pathogenesis of neuropathic pain (M. Zhang et al., 2022). While pro-inflammatory cytokines like TNF-α and IL-1β are known contributors to nociceptor sensitization, intensifying the perception of pain. Additionally, gabapentin exerts a modulatory influence on pro-inflammatory cytokines, including TNF-α and IL-1β, thereby dampening their role in exacerbating neuroinflammation and amplifying pain sensitivity. Our findings demonstrate that the levels of IL-1β and TNF-α were assessed in the DRG and spinal tissue of neuropathic rats. Combined chemotherapy treatment led to a significant increase in the levels of IL-1β and TNF-α in the DRG and spinal tissues, underscoring the neuro-inflammatory perturbations associated with CINP. Notably, gabapentin treatment at a dose of 60 mg/kg i.p. effectively mitigated these increases, highlighting its potential to restore neuro-inflammatory balance. (**Figure 4.16 A1-A2 & B1-B2**). These results collectively demonstrate that combined chemotherapy instigates a significant upregulation of neuro-inflammatory markers in DRG and spinal tissues, while gabapentin treatment successfully counters these neuro-

inflammatory changes, presenting a promising avenue for managing CINP-associated neuropathic pain.

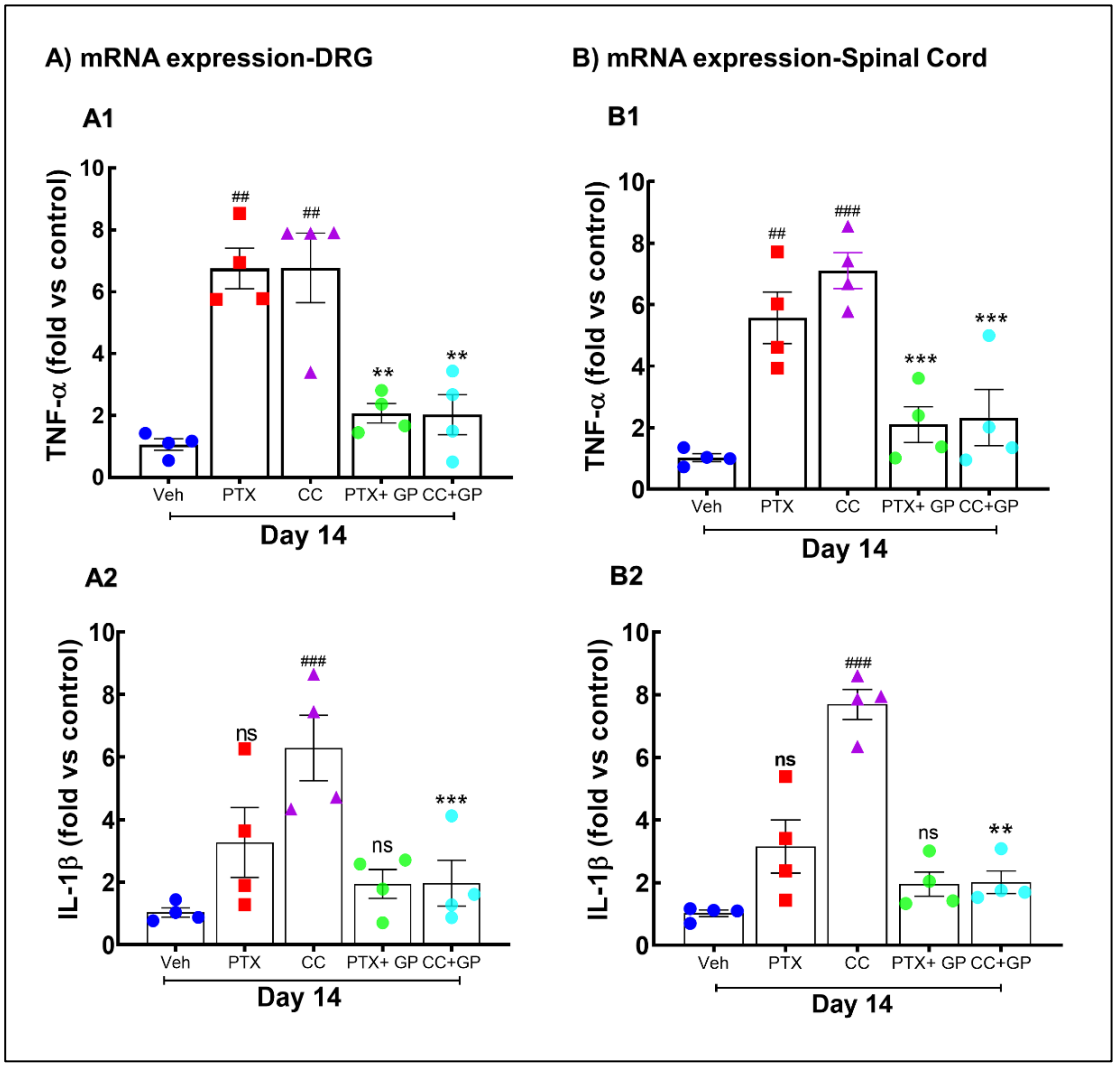

Figure 4.16 Effect of gabapentin on chemotherapy-induced neuro-inflammatory markers in rats. Both CC and PTX treatment causes a significant increase in the mRNA expression of TNF-α and IL-β levels in both DRGs and the lumbar region of the spinal cord which was significantly attenuated with gabapentin administration at day 14 but IL-β levels were not affected due to PTX administration and gabapentin treatment did not had any effect on the same. **(A1-A2)** TNF-α, and IL-1β mRNA expression in DRG. **(B1-B2)** TNF-α and IL-1β mRNA expression in spinal cord. Data were presented as mean ± SEM. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ indicates statistical significance as compared to the vehicle-treated rats. ***p<0.001and **p<0.01 indicates statistical significance as compared to the vehicle and PTX-treated rats. p<0.05 was considered statistically significant. n=4 biological and n=3 technical replicates.

4.3.5.9 Gabapentin treatment alleviated oxidative stress and increased antioxidant enzyme activity in sciatic nerve of neuropathic rats

Both models of chemotherapy significantly elevate the levels of malondialdehyde (MDA) in the sciatic nerve of CINP rats on the 14th day following the last chemotherapy injection. This increase in MDA, indicative of lipid peroxidation and oxidative stress, is consistent with the neurotoxic effects of chemotherapy. Statistical analysis using one-way ANOVA, followed by Tukey's multiple comparison test, demonstrates a notable effect across the groups on MDA level in the sciatic nerve [$F_{5,24}=45.24$; $p<0.001$, n=4] for both combined chemotherapy and PTX administration models [$F_{5,34}=85.24$; $p<0.001$, n=4]. The similar trend was also observed for nitrite levels in the sciatic nerves of both chemotherapy models compared to the vehicle treated group. One-way ANOVA followed by Tukey's multiple comparison test reveals a significant effect across the groups on nitrite activity in the sciatic nerve [$F_{5,36}=75.24$; $p<0.001$] for both chemotherapy models [$F_{5,26}=95.24$; $p<0.001$]. However, administration of gabapentin effectively mitigates the elevated levels of MDA and nitrite in rats subjected to combined chemotherapy and PTX treatment, in comparison to those receiving PTX or combined chemotherapy alone **(Figure 4.17 A & D).** Furthermore, in both chemotherapy models, a significant reduction in the enzymatic activity of glutathione (GSH), a major antioxidant enzyme, is observed in the sciatic nerve on the 14th day following the last chemotherapy injection, underscoring the impact of chemotherapy-induced oxidative stress. One-way ANOVA followed by Tukey's multiple comparison test demonstrates a substantial effect across the groups on GSH activity in the sciatic nerve [$F_{4,30}=125.57$; $p< 0.001$] for both PTX and combined chemotherapy-treated rats compared to vehicle-treated rats. Remarkably, administration of gabapentin at a dose of 60mg/kg significantly restores GSH levels in the sciatic nerve of CINP rats, effectively counteracting the GSH reduction induced by chemotherapy. A similar trend is observed in the levels of another crucial antioxidant

enzyme, superoxide dismutase (SOD), in the sciatic nerve. One-way ANOVA followed by Tukey's multiple comparison test reveals a significant effect across the groups on SOD activity in the sciatic nerve [$F_{5,35}=125.57$; $p<0.001$] for both PTX and combined chemotherapy-treated rats compared to vehicle-treated rats **(Figure 4.17 C).** Notably, gabapentin administration at a dose of 60 mg/kg, i.p. significantly restores GSH and SOD levels when compared to rats receiving only PTX or a combination chemotherapy **(Figure 4.17 B & C)**. These findings highlight the potential of gabapentin to ameliorate chemotherapy-induced oxidative stress in the sciatic nerve, offering a promising avenue for mitigating neurotoxicity associated with CINP.

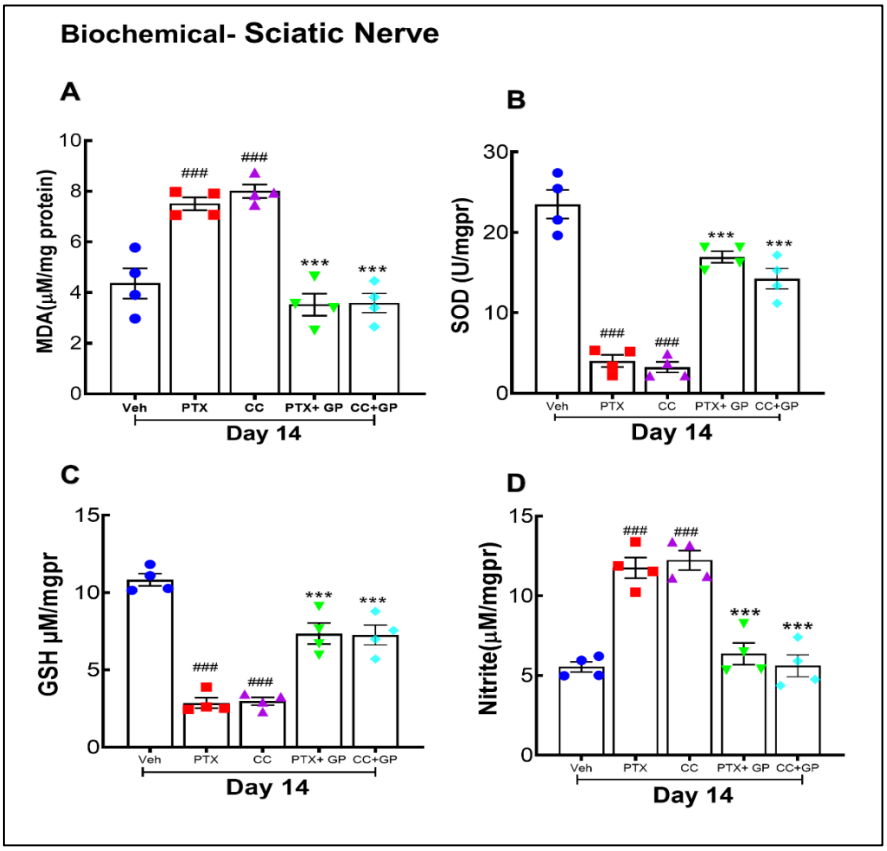

Figure 4.17 Effect of gabapentin on chemotherapy-induced biochemical changes in rats. Biochemical alterations: Chemotherapy-treated rats showed significantly decreased MDA and SOD levels in the sciatic nerve of rats, which were significantly restored post GP at 60 mg/kg i.p (A-B). GSH and nitrite levels in the sciatic nerves were significantly increased and was suppressed by gabapentin **(C-D).** Data were presented as mean ± SEM. $^{###}p<0.001$ indicates statistical significance as compared to the vehicle-treated rats. ***$p<0.001$ indicates statistical significance as compared to the vehicle

and PTX-treated rats. $p<0.05$ was considered statistically significant. n=4 biological and n=3 technical replicates.

4.3.6 Overall Summary and key findings

This study introduced a novel rat model for combined chemotherapy-induced neuropathic pain revealing prolonged hypersensitivity compared to conventional paclitaxel-induced neuropathy. Our novel model exhibited increased expression of TRP channels, pro-inflammatory cytokines, and neuropeptides, with notable NR2B upregulation. This clinically mimicable model provides a promising platform for evaluating CINP-targeted analgesics, bridging the therapeutic gap in neuropathic pain research. The working hypothesis and overall summary was shown in the **Figure 4.18.**

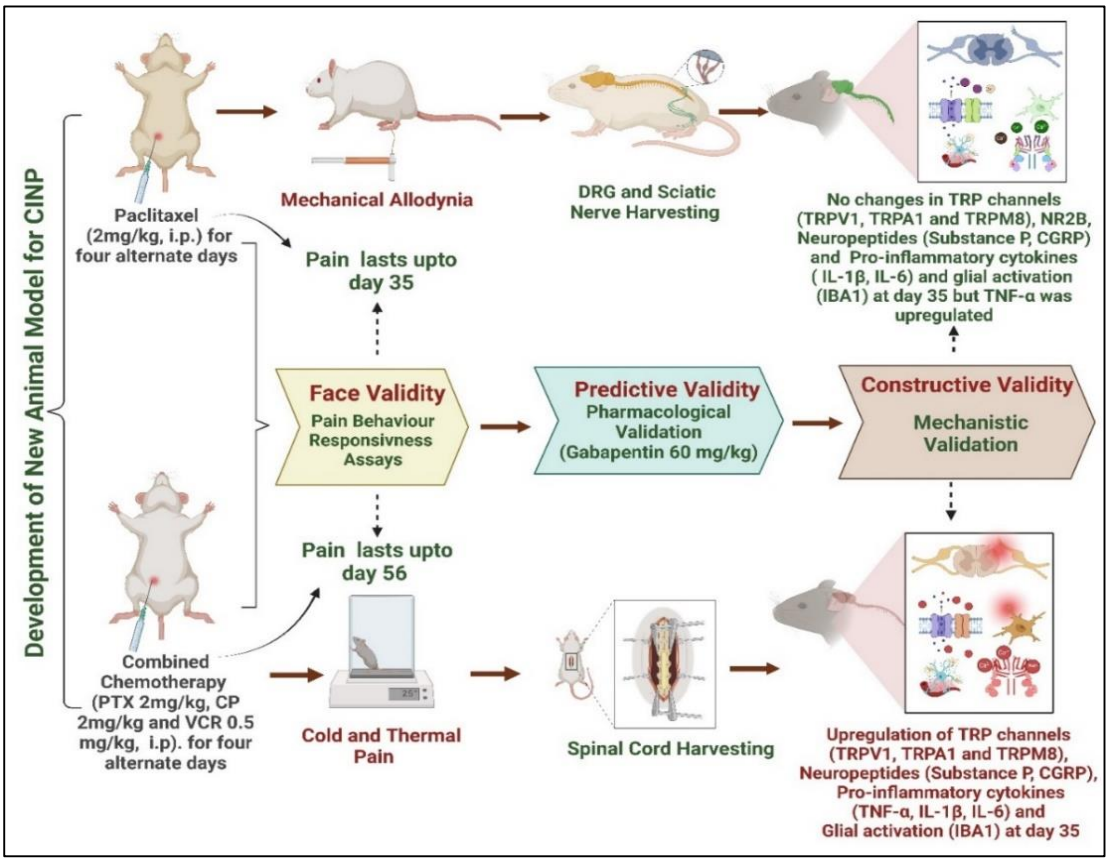

Figure 4.18. Working hypothesis and overall summary of the results. A novel chemotherapy-induced neuropathic pain (CINP) model using combined chemotherapy (CC) is established and validated. The CC model exhibits prolonged hypersensitivity and enhanced resilience with significant upregulation of TRP channels, NR2B,

neuropeptides, inflammatory markers and glia cell activation as compared to paclitaxel alone model. CC model successfully demonstrated the standard pharmacological paradigm as measured by using face, predictive and constructive validity assays. PTX: Paclitaxel, CP: Cisplatin, VCR: Vincristine.

4.4 Outcomes

In summary, we successfully developed and validated a novel animal model of chemotherapy-induced neuropathic pain using a clinically relevant combination of chemotherapeutic agents. This model represents a better alternative to the existing CINP models employing monotherapy and could help to expedite the analgesic drug-discovery process. Bridging the gap between bench and bedside may contribute to the translation values for CINP research.