

**CHAPTER 3:  
Materials &  
Methods**

## Materials & Methods

### 3.1 Drugs, chemicals and antibodies

The list of drugs, chemicals and antibodies used in this work along with their catalog number and source of procurement, are as follows:

**Table 3.1** List of drugs, chemicals and antibodies

S. No	Drugs/chemical/antibodies	Source
1.	Loperamide	TCI chemicals
2.	DALDA	Phtd Peptides (China)
3.	CB-13	Cayman (U.S)
4.	Morphine	Sir Sunderlal Hospital Pharmacy (Banaras Hindu University), Varanasi, India
5.	Gabapentin	Sigma-Aldrich (St. Louis, MO, USA)
6.	Paclitaxel	Sarabhai Chemicals India Pvt Ltd
7.	$\beta$ -actin antibody (ACTN05) (C4)	Abcam (USA)
8.	TRPA1 (MBS9612028)	MyBioSource
9.	Nav1.7 (14573)	Cell Signaling technology
10.	NR2B antibody (ab28373)	Abcam (USA)
11.	NF- $\kappa$ B antibody (sc-8008)	SantaCruz Biotechnology (U.S.A)
12.	IBA1 (SC32725)	SantaCruz Biotechnology (U.S.A)
13.	ICAM1 (SC8439)	SantaCruz Biotechnology (U.S.A)

14.	Tumour Necrosis Factor alpha (TNF- $\alpha$ ) (sc-52746)	SantaCruz Biotechnology (U.S.A)
15.	Interleukin-1 beta (IL-1 $\beta$ ) (sc-52012)	SantaCruz Biotechnology (U.S.A)
16.	Rabbit Anti-Mouse IgG H&L (#ab6728)	Abcam (USA)
17.	Goat anti-rabbit IgG H&L (HRP) (ab6721)	Abcam (USA)
18.	TriZol	Thermo scientific (USA)
19.	Maxima SYBR Green/Fluorescein qPCR Master Mix (#K0241)	Thermo scientific (USA)
20.	RevertAid first-strand cDNA synthesis kit (#K1622)	Thermo scientific (USA)
21.	Nuclease Free Water	Sigma-Aldrich (St. Louis, MO, USA)
22.	Bovine Serum Albumin (BSA)	Sisco Research Laboratories Pvt Ltd (Mumbai)
23.	Bradford reagent	Sigma-Aldrich (St. Louis, MO, USA)
24.	Enhanced chemiluminescence reagent	Biorad (USA)
25.	(5,5'-dithio bis-(2-nitrobenzoic acid)	Sigma-Aldrich (St. Louis, MO, USA)
26.	PVDF membrane	Merck Millipore
27.	Transfer buffer	Biorad (USA)
28.	Malondialdehyde	Sisco Research Laboratories Pvt Ltd (Mumbai)
29.	Griess reagent	Sisco Research Laboratories Pvt Ltd (Mumbai)
30.	Glutathione	Sigma-Aldrich (St. Louis, MO, USA)
31.	Acetone	Sisco Research Laboratories Pvt Ltd (Mumbai)
32.	Chloroform	Sisco Research Laboratories Pvt Ltd (Mumbai)
33.	Ethanol	Sigma-Aldrich (St. Louis, MO, USA)

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34.	Tris-hydrochloric acid	Sisco Research Laboratories Pvt Ltd (Mumbai)
35.	EDTA	Sigma-Aldrich (St. Louis, MO, USA)
36.	Disodium hydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
37.	Potassium dihydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
48.	Potassium chloride	Sisco Research Laboratories Pvt Ltd (Mumbai)
39.	PMSF	Sigma-Aldrich (St. Louis, MO, USA)
40.	2- mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
41.	Bromophenol blue dye	Sigma-Aldrich (St. Louis, MO, USA)
42.	Sodium dodecyl sulphate (SDS)	Sisco Research Laboratories Pvt Ltd (Mumbai)
43.	Ammonium persulphate	Sisco Research Laboratories Pvt Ltd (Mumbai)
44.	Tetramethylethylenediamine (TEMED)	Sisco Research Laboratories Pvt Ltd (Mumbai)
45.	Glycerol	Sisco Research Laboratories Pvt Ltd (Mumbai)
46.	Tris-base	Sisco Research Laboratories Pvt Ltd (Mumbai)
47.	Glycine	Sisco Research Laboratories Pvt Ltd (Mumbai)
58.	Prestained protein ladder	Genetex biotech (India)
49.	Protease inhibitor cocktail- P8340	Sigma-Aldrich (St. Louis, MO, USA)
50.	Sodium chloride	Loba Chime (India)
51.	Sodium fluoride	Sigma-Aldrich (St. Louis, MO, USA)
52.	Triton x100	Loba Chime (India)
53.	Sodium orthovanadate	Loba Chime (India)
54.	Sodium deoxycholate	Loba Chime (India)

55.	Acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
56.	Bis-acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
57.	HCL	Sigma-Aldrich (St. Louis, MO, USA)
58.	NaOH	Sigma-Aldrich (St. Louis, MO, USA)

### 3.2 Equipment and software

Following is the list of equipment and software along with their make, utilized in the present study:

**Table 3.2** List of equipment & software:

S. No	Equipment/software	Source
1.	Chemidoc	Biorad (USA)
2.	Gel electrophoresis assembly	Biorad (USA)
3.	Transblot	Biorad (USA)
4.	Nanodrop	Thermo scientific (USA)
5.	Rotor-Gene Q 2plex HRM real-time PCR System	Qiagen (Germany)
6.	pH meter	Eutech (UK)
7.	Cold centrifuge	Eppendorf (Germany)
8.	Microplate reader SpectraMax M5	Molecular Devices (USA)
9.	Micropipettes	Eppendorf (Germany)
10.	Refrigerator (4°C)	Remi (India)
11.	Deep freezer (-40°C)	Remi (India)
12.	Deep freezer (-80°C)	Thermo scientific (USA)
13.	Millipore system	Merk-Sigma (USA)
14.	Tissue homogenizer (MT-30K)	MIULAB (China)

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<b>15.</b>	Hargreaves apparatus	Ugo Basile (Italy)
<b>16.</b>	Von-Frey filaments	Anesthesio (USA)
<b>17.</b>	Conditioned Place Preference Apparatus	Rolex (India)
<b>18.</b>	Rongeur	Fine Science Tools (USA)
<b>19.</b>	Temperature sensor	Aptech Deals (Indian)
<b>20.</b>	Von-Frey mesh	Workshop IIT (BHU), Varanasi
<b>21.</b>	Dry Bath	Precious Instrument techno (Delhi, India)
<b>22.</b>	Rocker	Precious Instrument techno (Delhi, India)
<b>23.</b>	Vortex	Remi (India)
<b>24.</b>	Spinwin	Abdos (India)
<b>25.</b>	Weighing balance	Sartorius (Germany)
<b>26.</b>	Surgical tools	Bharat Surgicals (Varanasi, India)/ Fine Science Tools (USA)
<b>27.</b>	Matlab software	MathWorks (USA)
<b>28.</b>	Microsoft word	Microsoft (USA)
<b>29.</b>	Microsoft power point	Microsoft (USA)
<b>30.</b>	Microsoft excel	Microsoft (USA)
<b>31.</b>	Chemdraw Software	PerkinElmer (USA)
<b>32.</b>	Image Lab software	Biorad (USA)
<b>33.</b>	Rotor-Gene Q Series software	Qiagen (Germany)
<b>34.</b>	GraphPad Prism 8.0	GraphPad (USA)

### **3.3 *In-vivo* studies**

#### **3.3.1 Experimental animals**

Male Sprague Dawley rats (200-250 gm) were used in the study. Animals were housed four per cage in standard housing conditions; temperature  $21 \pm 2^{\circ}\text{C}$ ; 12-hour light-dark cycle. Animals were provided with standard laboratory food *ad-libitum* and sterile water. All the animals were randomly assigned to the different experimental groups (n=8/group).

#### **3.3.2 Ethical committee approval**

All experiments were performed in accordance with the guidelines by International Association for The Study of Pain and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi. The Institutional Animal Ethics Committee of Indian Institute of Technology, Banaras Hindu University, Varanasi approved all the experimental protocols with approval no. IIT(BHU)/IAEC/2022/014.

#### **3.3.3 Paclitaxel Induced Chronic Pain Model**

CINP was developed using paclitaxel model [15,241]. Paclitaxel was diluted using 0.9% normal saline to make a working concentration of 2mg/ml, from its commercially available concentration of 6mg/ml. Initially, the rats were acclimatized in the test room for about 2-3 days to get habituated with the environment, 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. Rats were administered the solution of

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paclitaxel at a dose of 2 mg/kg through intraperitoneal (i.p) route on day 1, day 3, day 5, day, 7 to obtain a cumulative dose of 8 mg/kg as previously described [150]. Twenty-eight days post first paclitaxel injection i.e., once the pain response peaked, animals were treated with different doses of MOR and CBR agonists (as per study), 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.

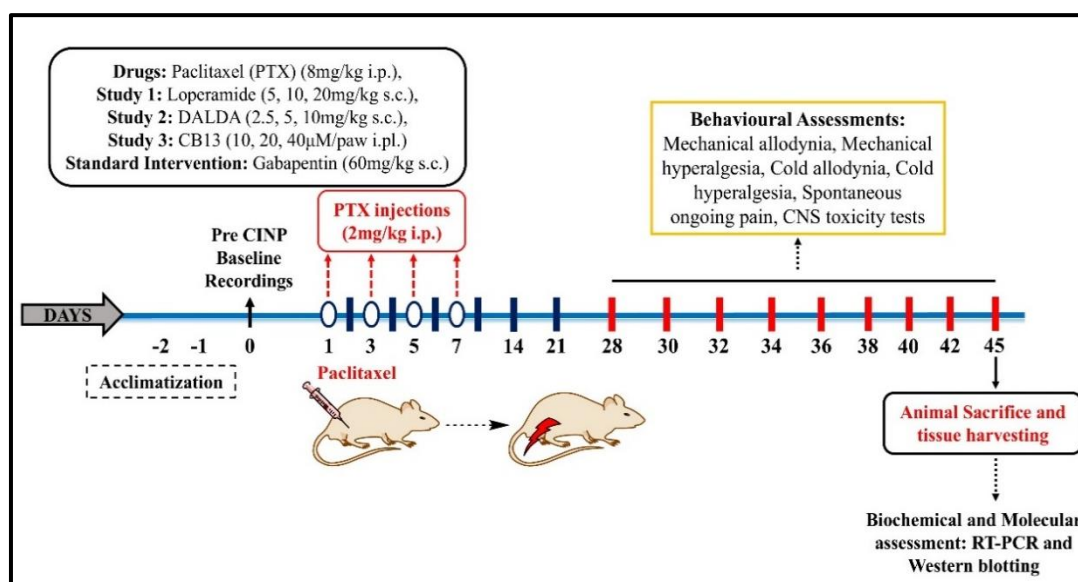


Figure 3.1 Experimental timeline of the study.

### **3.3.4 Behavioural Analysis**

#### **3.3.4.1 von-Frey hair Test: Mechanical allodynia**

von-Frey hair test was used to assess the dynamic mechanical allodynia in neuropathic rats. The mechanical sensitivity was assessed by the application of von-Frey filaments (Anesthesio, USA) of different forces ranging from 0.40-15gm on the dorsal surface of hind paws [242,243]. Animals were placed in the plexiglass chambers on the top of the wire-mesh table and the von-Frey filaments were applied in the sub plantar region of rat's paws from the bottom of the mesh. The up-down method was used to measure the mechanical sensitivity before and after nerve injury and post-drug treatment in rats. von-Frey hairs were applied at the middle of the sub-plantar region of the hind paw of rats. Paw withdrawal, licking, and shaking of the paw in response to von-Frey filament was considered as a positive response. The test was started with a lower force of 1.8 gm filament. If there was no response, then filament of higher force was used. When the response was positive then testing with lower force filament was done. The test was performed till five positive responses were observed or the test reaches the withdrawal maximum and the minimum threshold at higher or lower forces [88]. Pre-injury baseline and 0.5 hr, 1 hr, 2 hr and 4hr, post-drug/vehicle administration thresholds were measured in PTX treated neuropathic rats. The series of responses were further quantified using the MATLAB software.

#### **3.3.4.2 Pinprick test: Mechanical Hyperalgesia**

Mechanical hyperalgesia was evaluated using pinprick test. The rats were habituated on a wire mesh for 2-3 days, followed by providing stimulus with a custom-designed pin-prick stimulator prepared by attaching a 22-gauge needle perpendicularly

on a straight stick. The pin was used to evoke noxious response in hind paw, taking care not to pierce the skin of the paw. The test was repeated 10 times for each hind paw and the frequency of paw withdrawal per 10 trials was recorded [243,244].

### **3.3.4.3 Acetone evaporation test: Cold Allodynia**

Acetone spray test was used to assess cold allodynia in rats. The test involves placing the animals in plexiglass chambers over a wire mesh and spraying acetone on the plantar surface of the rats' hind paw. Acetone is innocuous in nature and produces a cooling sensation on the skin having temperatures between 15–21°C. Scoring was given to the animal responses as per the following scale: 0-No response, 1- flicking of paw, 2- repeated flicking of paw, 3- repeated flicking along with licking of paw [245]. The average of three trials was recorded as the response score by a blinded experimenter.

### **3.3.4.4 Ice floor test: Cold Hyperalgesia**

Cold hyperalgesia in rodents was assessed using ice floor method as per the previously published protocol from our lab [246]. The animal was placed on an ice-floor and a temperature sensor was used to maintain the temperature between 0-4°C. The animal behavior was video recorded for 1 min and the number of paw lifts were counted by the blinded experimenter.

### **3.3.4.5 Conditioned place preference: Spontaneous ongoing pain assay**

A conditioned place preference (CPP) apparatus, consisting of two large chambers connected by a central corridor that allows free movement between the chambers, was used to study the preference for the test drug, which indicates relief from spontaneous ongoing pain behavior. The two large chambers were differentiated with

different visual and tactile cues such as black and white stripes or triangles on the walls of the chambers and the flooring with or without grids, allowing animals to differentiate the two chambers. Any Maze software (Version 7.1 Stoelting, USA) was used to video-track and analyze the time spent by rats in each of these chambers [247,248]. Animals were acclimated in the CPP apparatus for 20–30 minutes after being habituated in the test room. We recorded preconditioning baselines for each rat the next day in order to gauge their compartment preference before drug treatment. Animals were free to explore every compartment during preconditioning, and video tracking was conducted for 15 minutes to determine the time spent in each compartment. Only rats that preferred either of the chambers by more than 20% or less than 80% were considered for further experiments [247]. Further, conditioning was done over the course of two days in two sessions each day. Animals were restricted to the vehicle paired chamber for 30 minutes during the first session. A second session was conducted four hours later, during which animals were administered with the test drug and confined to the paired chamber for 30 minutes. On the next day during post-conditioning, rats were given unrestricted access to all the compartments for 15mins. The post-conditioning trial was video recorded, and the amount of time the rats spent in each compartment was calculated and analyzed to determine their preference for the test drug.

### **3.3.5 Behavioral neurotoxicity assays**

#### **3.3.5.1 Open field test**

Impaired locomotor behavior, post drug treatment indicates neurotoxic potential of the drug. We used the open field test to study the locomotor activity of rats by measuring the total distance travelled and average speed. The method used has been

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outlined in our previous studies [248]. Rats were allowed to acclimatize and get familiarized with the apparatus one day before the test. Rats were placed in the open field apparatus (45x45x75cm) on the testing day, and the session was recorded for 10 minutes, using the Any Maze software (Version 7.1 Stoelting, USA).

### **3.3.5.2 Rota-rod test**

Development of motor-incoordination after drug treatment is an indication of neurotoxic potential of the drug. Here, we used the rota rod apparatus to measure the effect of drug on motor-coordination in rats. The testing protocol described in previous studies was adhered to. It included a training session and testing after that. Rats were initially trained for 120 seconds at a speed of 5 revolutions per minute (rpm). After training, the test was conducted at a constant speed of 25 rpm before and after drug administration. Rats were allowed to stay on the accelerating rod for a maximum of 120 seconds, and the time spent on rod was recorded [242,249].

### **3.3.6 Tissue harvesting and storage**

Euthanasia was performed after completion of the behavioral studies and the animals were sacrificed humanely using deep isoflurane anesthesia followed by cervical decapitation. Lumbar L4-L5 DRGs were dissected out by cutting through the muscles and pulling out the transverse processes of the spinal cord [221,243]. Further, the deep cut was made through the skin above the spinal cord. The vertebral column was removed using a rongeur and Dumont forceps and the spinal cord was exposed fully. The L4-L5 lumbar region was identified and collected. The sciatic nerve was also harvested by dissecting through the bicep femoris muscle. All tissues were stored at -80°C for further biochemical and molecular analysis.

### **3.3.7 Biochemical assays**

#### **3.3.7.1 Lipid peroxidation**

This assay is used to estimate the levels of malondialdehyde which is the end product of peroxidation of lipids. It was performed using thiobarbituric acid reactive substances (TBARS) assay method [250,251]. The supernatant was added to 20% Acetic acid, 0.8% TBA (thiobarbituric acid) and 8.1% SDS. This mixture was vortexed and then incubated in a water bath at 95°C for 1hour. After allowing the mixture to cool, 200µl was taken in duplicates in a 96 well plate and absorbance was taken at 532nm using multimode microplate reader.

#### **3.3.7.2 Nitrite estimation**

Griess reagent was used to measure the nitrite levels which indicate the nitrosative stress inside a cell. Equal amounts of tissue lysate were mixed with the Griess reagent and incubated for 30 mins at room temperature. The absorbance was taken at 540nm using a microplate reader [221]. Levels of nitrite per mg of protein were calculated using the equation that has been derived from the standard curve.

#### **3.3.7.3 Glutathione estimation**

Ellman's reagent (DTNB) was used to measure glutathione levels. DTNB (di thio bis nitro benzoic acid) reacts with the reduced form of glutathione in the tissue lysate and gets converted into a yellow-coloured compound i.e., TNB (thio nitro benzoic acid) whose absorbance was measured at 412nm [243,252]. Desired volume of sample was taken to which phosphate buffer (pH-7.4) and 0.1mM DTNB (prepared in pH 8.0 phosphate buffer) were added. This mixture was vortexed and left for incubation at 48-

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50°C for 30mins. Later, 200µl of sample was taken in duplicates in a 96 well plate and absorbance was taken at 412nm using multimode microplate reader.

### **3.3.8 Molecular biology studies**

#### **3.3.8.1 Western blot analysis**

DRG and spinal cord tissue lysates from rats were prepared using radioimmunoprecipitation assay (RIPA) buffer. The estimation of protein in each tissue sample was done by the Bradford method. Briefly, the 198µl of Bradford reagent was added to the 2µl of sample in 96 well plates followed by 10min incubation at room temperature and reading was taken at 595nm using a multimode plate reader. The protein concentration in each sample was determined using the standard curve plotted with bovine serum albumin (BSA).

**Table 3.3 Composition of RIPA buffer**

<b>S.No.</b>	<b>Chemical</b>	<b>Concentration</b>
1	Tris-HCL (pH 8.0)	50mM
2	NaCl	150mM
3	Triton X	0.1%
4	Sodium deoxycholate	0.3%
5	SDS	0.1%
6	Sodium fluoride	1mM
7	Sodium orthovanadate	1mM
8	EDTA	2mM
9	EGTA	2mM
10	PMSF	1mM
11	Protease inhibitor	5µl/100 mg of tissue

The samples were prepared by mixing the 30µg of protein with loading buffer (Recipe shown in table 3.4) and volume was made up to 20µl with deionized water. Next, the samples were heated on a dry bath at 100°C for 3 mins and briefly centrifuged at room temperature. The gel was set for electrophoresis and an equal amount of sample (20µl) and protein ladder (2.5µl) were loaded into the wells of polyacrylamide gel and protein were allowed to separate using Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with running buffer (Recipe is given in table 3.5). The initial voltage was maintained at 70 V till the lanes reached the resolving buffer and then made run at 90 V.

**Table 3.4 Loading buffer recipe**

S.No.	Composition	Concentration
1	SDS	4%
2	2-mercaptoethanol	10%
3	Glycerol	20%
4	Bromophenol blue	0.004%
5	Tris HCl	0.125 M

**Table 3.5 Running buffer recipe**

S.No.	Composition	Concentration
1	Tris	25mM
2	Glycine	190mM
3	SDS	0.1%

After the gel electrophoresis, the proteins were transferred to nitrocellulose/PVDF membranes using transfer buffer (Biorad, USA). Briefly, the transfer stacks and nitrocellulose/PVDF membranes were soaked into the transfer buffer and a sandwich was prepared. The cassettes were loaded into the transblot system and protocol was made run 0.7amp, 15V for 45 mins. After the completion of the

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protein transfer, the membranes were blocked to reduce the nonspecific binding using 3% BSA solution prepared in TBST (tris buffer saline-tween-20) over the rocker system for 2hr. Membranes were washed with TBST for 3X using an orbital shaker for 5 min each. Further, the membranes were incubated with primary antibodies at 4°C for overnight. Following incubation, membranes were washed with TBST and incubated with their respective secondary antibodies for 1hr. Immune complexes formed were detected using ECL (chemiluminescent agent) (Invitrogen, Carlsbad, California, United States) [253,254]. Blots were visualized in the gel documentation system (ChemiDoc™, BioRad, Hercules, California, United States) and quantified using Image J software.

### **3.3.8.2 Reverse transcription polymerase chain reaction (RT-PCR) analysis**

Total RNA was isolated from the spinal cord and DRG tissues using the Trizol reagent method. Briefly, trizol was added to the samples, and tissue was homogenized using an automated homogenizer at an ice-chilled platform. The resulting mixture was kept at room temperature for 10 mins and then chloroform was added followed by centrifugation at 12000g at 4°C. Next, the supernatant was removed and an equal amount of ethanol was added to the same followed by incubation for 10 min and centrifugation at 12000g at 4°C for 15 min. The RNA pellet was observed and washed with 75% ethanol twice. Finally, the resulting pellet was dissolved in nuclease-free water and RNA was quantified using NanoDrop (Thermo Scientific).

Further, cDNA was prepared using commercially available cDNA Synthesis kit (K1622 Thermo scientific) by mixing the template RNA, random primers, reaction buffer, RNase inhibitor, dNTP mix, revert aid RT and final column was made up using

nuclease-free water. The cDNA synthesis reaction was made run for 60 min at 45°C and terminated at 70°C for 5 min. A reaction master mix of 15µl was prepared by mixing Maxima SYBR Green/Fluorescein qPCR Master Mix (K0241), forward primer, reverse primer, template DNA, and nuclease-free water (Sigma W4502) [255]. The run was performed on Rotor-Gene Q (Qiagen, Germany). Table 3.6 shows the list of used primers for RT-PCR

**.Table 3.6 Primers used in RT-PCR analysis**

<b>S.No.</b>	<b>Gene</b>	<b>Sequence</b>	<b>Primer 5'&lt;-----Sequence-----&gt;3'</b>
1	GAPDH	Forward	CAGTGCCAGCCTCGTCTCAT
		Reverse	CAAGAGAAGGCAGCCCTGGT
2	TRPM8	Forward	AGAAGCTCTTGGCTGTTTGAGC
		Reverse	TTTCTGCGGCTCCTCATGCT
3	Nav1.8	Forward	CAAGGAGAACCACGGGGACTT
		Reverse	TTTGGACTTGTGGCAACTGCTC
4	Cav2.2	Forward	TCCAGCGTAAACTCACCGCA
		Reverse	TGGAGCCTTAGCTGACTGGC
5	Cav3.2	Forward	CTGGAGAGGAGGCGCAGG
		Reverse	GCACAGCGAATGGATGGAGC
6	TNF $\alpha$	Forward	GTAGCCCACGTCGTAGCAAAC
		Reverse	ACCACCAGTTGGTTGTCTTTGA
7	CGRP	Forward	GGCTGTTCTCTGGGCTATTATG
		Reverse	CATCTTCCTGGGTGATTCTCTTT
8	Sub-P	Forward	TGTGGCTGGCCATGAGTTCT
		Reverse	CCCAGACGGAACCTGTCGTT
9	IL6	Forward	TCTGGTCTTCTGGAGTTCCGTT
		Reverse	GAGAGCATTGGAAGTTGGGGT

### **3.3.9 Statistical analysis**

Behavioural data was analyzed using two-way ANOVA with post hoc Bonferroni's multiple comparisons. One-way ANOVA followed by Tukey's multiple comparison test was used for analysis of molecular studies data. Microsoft excel was used to interpret the raw data of RTPCR and western blot by calculating the fold change and relative expressions respectively. GraphPad Prism 8.0.2 was used to perform the statistical analysis. Data was presented as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant.