Pharmacology, Biochemistry and Behavior 91 (2009) 417–422

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Increased hyperalgesia by 5-nitro-2, 3-(phenylpropylamino)-benzoic acid (NPPB), a chloride channel blocker in crush injury-induced neuropathic pain in rats

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ARTICLE INFO

Article history:
Received 21 February 2008
Received in revised form 26 July 2008
Accepted 12 August 2008
Available online 23 August 2008

Keywords:
Neuropathic pain
Chloride channel
Ivermectin
5-nitro-2, 3-(phenylpropylamino)-benzoic acid (NPPB)

ABSTRACT

Chloride channels belong to diverse group of anion selective channels involved in different signaling processes. The present study was planned to investigate the involvement of chloride channels in crush injury-induced neuropathic pain in rats by using ivermectin, a ligand gated chloride channel opener and NPPB, a CaCC blocker. The effect of ivermectin (5, 10, 20 mg/kg i.p. or 50, 100 and 200 μg/rat by i.c.v. route) and NPPB (10, 20 and 40 mg/kg i.p.) was investigated on pain behavioural thresholds in crush injury-induced neuropathic pain rat model. Reduction in pain threshold by mechanical, thermal and cold stimuli confirmed the development of neuropathic pain in rats after crush injury. Ivermectin administered either by i.p. or i.c.v. route did not alter the pain threshold in mechanical, thermal and, cold allodynia tests in rats. NPPB (20 and 40 mg/kg i.p.) significantly reduced the pain threshold crush injury neuropathic pain model suggesting its hyperalgesic effect. The results showed that NPPB increased significantly the mechanical and thermal hyperalgesia in crush injury-induced neuropathic pain rat model, whereas ivermectin, either by i.p. or i.c.v. route of administration, has no effect on pain symptoms in this model. NPPB hyperalgesic effect is independent of CaCCs inhibition and may be due to blockade of Ca^{2+}-activated K^+ channel.

1. Introduction

Convincing evidence from animal and human study shows that the starting point of neuropathic pain is a lesion of somatosensory system (Zimmermann, 2001; Jensen and Baron, 2003). Injured peripheral nerve fibres give rise to an intense and prolonged input of ectopic activity to central nervous system (CNS) and in some cases also secondary changes of excitability of dorsal horn neurons (Zimmermann, 2001; Jensen and Baron, 2003). There is great deal of information about the important role played by sodium, calcium and potassium ion channels in neuropathic pain, whereas the role (s) played by chloride (Cl^-) channels remained uninvestigated (Waldegger and Jentsch, 2000; George et al., 2001). (Cl^-) channels are functionally and structurally diverse group of anion selective channels involved in various physiological processes. Chloride channels are subdivided according to their gating mechanisms: ligand gated chloride channel (GABA or glycine), voltage gated Cl^- channel (ClC), the phosphorylation regulated cystic fibrosis transmembrane conductance regulator (CFTR) channel, volume regulated anion channels (VRACs), and calcium-activated chloride channels (CaCCs). The first three types of chloride channel are well understood with respect to molecular identity, channel properties and physiological roles. Inhibitory neurotransmission in mammalian CNS is mediated primarily by GABA and glycine. Glycine is predominantly involved in spinal cord and brain stem, whereas GABA is more commonly involved in brain. Ivermectin is a macrocyclic lactone, widely used as antiparasitic agent in domestic animals and is considered as drug of choice for filariasis and onchocerciasis in humans (Ottesen et al., 1999). Ivermectin, a chloride channel opener, acts as aniticonvulsant in variety of seizure models including mice and its action seems to be mediated by $GABA_A$ receptors (Dawson et al., 2000). Ivermectin has also been known to displace [^{3}H] strychnine in radiolabeled binding studies, implying that it may exert some effect on glycine receptor (GlyR) (Graham et al., 1982) have not been studied for pain/neuropathic pain.

CaCCs are expressed in a variety of neurons, including dorsal root ganglion (DRG) neurons, spinal cord and neurons from autonomic nervous system (Hartzell et al., 2005). Somatosensory neurons from DRG that sense skin temperature, touch, muscle tension and pain express CaCCs (Stapleton et al., 1994); CaCCs in DRG are responsible for after-depolarization following action potential (De Castro et al., 1997). Thus, opening of CaCCs by Ca^{2+} entry or Ca^{2+} release from intracellular stores would depolarize the cell membrane or produce after-depolarization (Duchen, 1990). The selective expression of calcium-activated chloride current within medium and large diameter neurons conditioned for rapid and efficient growth suggest that these channels play a specific role in post-injury behaviour of sensory neuron subpopulation such as, neuropathic pain and/or axonal regeneration (Hilaire et al., 2005). Calcium-activated chloride current-induced after-depolarization in axotomised sensory neuron is

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doi:10.1016/j.pbb.2008.08.017

regulated by K^+ current density (André et al., 2003). However, *per se*, effect of CaCCs blockers has not been studied for anti-hyperalgesic effect in neuropathic pain models.

The pharmacology of Cl^- channel blockers is plagued by low potency, low selectivity and tissue variability, but, Cl^- channel blockers are still used as probes for CaCCs in functional studies without due consideration of these limitations (Greenwood and Leblanc, 2006). In smooth muscle cells, several Cl^- channel blockers including fenamates and anthracene-9-carboxylic acid (A-9-C) activate large conductance Ca^{2+} activated K^+ channel (BkCa) at similar concentration required to block calcium-activated chloride current (IClca) (Toma et al., 1996). 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) has been found to block CaCCs in DRG neurons (Currie et al., 1995), *Xenopus* oocyte (Wu and Hamill, 1992) and pulmonary artery endothelium (Nilius et al., 1997; Piper et al., 2002). There appears to be no direct *in vivo* studies of CaCC modulators on pain and nociception. However, CaCC blockers flufenamic acid and niflumic acid have been found to attenuate δ-opioid receptor agonist [D-Ala2] deltorphin II suggesting that Ca^{2+}-activated Cl^- channel is, at least in part, involved in the production of δ-opioid receptor-mediated antinociception in the mouse spinal cord (Yamazaki et al., 2000).

Considering the suggested importance of Ca^{2+}-activated and ligand gated chloride channels in pain, nociception and convulsions, the present study was planned to investigate the effect of chloride channel modulators in crush injury-induced neuropathic pain in rats by using ivermectin, a chloride channel opener and NPPB, a chloride channel blocker.

2. Methods

2.1. Animals

Adult male (Wistar strain) rats (125–150 g) procured from Laboratory Animals Resource Section of Indian Veterinary Research Institute were used for the present study. The animals were housed in groups of 5–6 in polypropylene cages 7 days prior to surgical procedure, so as to acclimatize to the laboratory conditions. Balanced rat feed and water were provided *ad libitum* along with all necessary care throughout the experimentation. The rats were kept at room temperature of 25 ± 2 °C. The experimental procedures were approved by Animal Ethics Committee of the Institute.

2.2. Induction of crush injury-induced neuropathic pain in rats

Rats were anaesthetized by using ketamine hydrochloride at 100 mg/kg i.m. After achieving proper anaesthesia, hairs around mid-thigh region were clipped and shaved properly. The sciatic nerve was exposed at mid-thigh level after giving small incision through *biceps femoris* muscle. Mosquito artery forceps was applied proximal to the trifurcation of sciatic nerve for 30 s as illustrated in Fig. 1 (Bridge et al., 1994) and then it was removed. The muscle incision was closed by chromic absorbable suture (3–0) and the skin incision was closed with silk suture. Similar surgery performed on opposite (left) leg without applying mosquito artery forceps, was considered as sham operated. After suturing, povidone iodine solution was applied externally and prophylactically, oxytetracyclin (Terramycin, Pfizer, India) was administered at 50 mg/kg i.m. for 3 consecutive days to check secondary infection. After surgery, animals were kept individually in separate cages along with feed and water *ad libitum*. All aseptic conditions were provided throughout the experiment. Total 24 rats were used for each drug trial and each group comprised of six rats.

2.3. Intracerebroventricular (i.c.v.) cannulation

The experimental rats were cannulated i.c.v. (Verster et al., 1971) simultaneously under proper aseptic conditions after inducing crush injury. An incision was made over the mid-line of skull of about 3 cm approximately; from anterior to the posterior direction and a small burr hole was drilled at the coordinates, 2 mm lateral and 1 mm caudal towards the right hand side of the bregma mark over the skull. Two stainless steel screws were fixed to the skull taking care that they do not pierce the bone, one at 1 mm behind and the other 1 mm in front of the bregma mark. A 47 number polyethylene guide cannula, filled with artificial cerebrospinal fluid was inserted through the small hole to a depth of 4 mm below the skull surface. The cannula was fixed to the skull surface with the help of stainless steel screws and dental cement. The hold up capacity of the cannula was 5 μl. After fixing, 5 μl artificial cerebrospinal fluid (sodium chloride—138.50 mM, potassium chloride—3.35 mM, calcium chloride—1.26 mM, magnesium chloride —1.16 mM, sodium bicarbonate—21.0 mM, sodium dihydrogen orthophosphate—0.50 mM, urea—2.20 mM and dextrose—3.40 mM) was passed through the cannula into the right lateral ventricle to clear the passage. Finally, the top of the cannula was sealed by mild heat. The operated rats were allowed to recover for 7 days and divided into groups of six animals each for control and treatment group.

After the termination of the experiment, all the rats were injected i.c.v. with 5 μl if 1% Evan's blue solution in order to ascertain the correct position of the cannula in the right lateral ventricle of the brain which was 95–98% in the hands of trained person. The data presented pertains to the animals in which i.c.v. cannulation was correctly done.

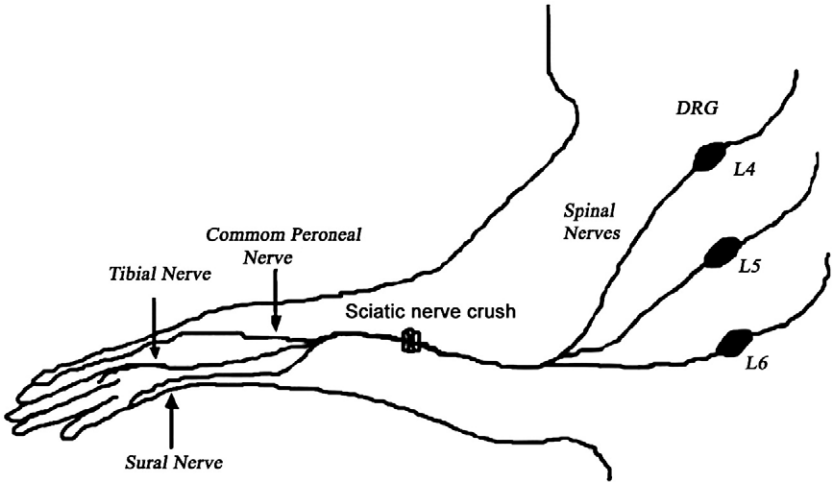

Fig. 1. Illustration demonstrating the anatomical location of the surgical intervention.

2.4. Drug administration

Effect of chloride channel modulators was investigated on 7th day following surgical procedure.

Ivermectin (Sigma-Aldrich) dissolved in propylene glycol was administered in crush injury-induced neuropathic rats at the dose rate of 5, 10 and 20 mg/kg i.p. and 50, 100 and 200 μg/rat by i.c.v. route and control rats were treated with propylene glycol. The doses of ivermectin were selected between 1/10th and 1/3rd of LD_{50} dose (55 mg/kg i.p.) as reported in the literature for rats (Material Safety Data Sheet, Merck AgVet, IVOMEC POUR-ON. http://www.compasnac.com/wpsdemo/docs/11/1140/1140008.pdf) and i.c.v. doses were 100th of the i.p. doses. In i.c.v. administration, ivermectin was dissolved in propylene glycol afresh just before its administration, by reconstituting it in sterile artificial cerebrospinal fluid. The concentration of the ivermectin in artificial cerebrospinal fluid was adjusted in such a way that a constant volume of 5 μl of the drug solution was injected i.c.v. by cutting the sealed top of the cannula at the rate of 1 μl/min, using a 10 μl syringe. Then, 5 μl of artificial cerebrospinal fluid was administered to push the full amount of the drug solution into the ventricles and the cannula was resealed. Untreated control groups received similar volume of vehicle in artificial cerebrospinal fluid.

NPPB (Sigma-Aldrich) dissolved in dimethyl sulphoxide (DMSO), was administered in crush injury-induced neuropathic rats at the dose rate of 10, 20 and 40 mg/kg i.p. based on preliminary exploratory experiments and control rats were treated with vehicle solution DMSO.

After drug administration, pain threshold in rats was recorded in drug-treated and vehicle-treated control rats.

2.5. Pain threshold assessment

Pain threshold in rats was recorded by mechanical and paw withdrawal latencies in thermal and cold allodynia tests at different time intervals. Pain withdrawal latencies/threshold were also determined before surgery. These tests were conducted blindly in the manner that the recorder did not know the nature of experimental manipulation. Pain threshold/paw withdrawal latency tests were done on the same day with the sequence of mechanical hyperalgesia, followed by thermal hyperalgesia and cold allodynia tests.

2.6. Mechanical hyperalgesia

The pressure in g was recorded by Randall–Selitto assay method (Randall and Selitto, 1957), using Randall–Selitto analgesiometer (Ugo Basile, Varese, Italy), immediately prior to the administration of ivermectin and NPPB (0 h) in crush injury-induced neuropathic pain in rats and at 1, 3, 5 and 7 h after drug administration. The change in pain threshold in test group was compared with that of vehicle-treated control group. Results are expressed as mean ± S.E.M.. A cut off pressure of 200 g was used to avoid tissue damage.

2.7. Thermal hyperalgesia

The latency to radiant heat was measured by radiant heat apparatus (Ugo Basile, Varese, Italy), immediately prior to (0 h) and at 1, 3, 5 and 7 h after drug administration. The paw was placed on the heat radiator with infrared intensity of lamp was set at 40 in a manner that the plantar surface of the affected paw touching the heat radiator without any apparent stress to the animal under test and the withdrawal time (s) of paw was recorded. The change in paw withdrawal latency of test group was compared with that of vehicle-treated control group. Results are expressed as mean ± S.E.M. A cut of latency of 20 s was used to avoid tissue damage. The mean paw withdrawal latency before surgery ranged between 3.88 ± 0.06 and 5.20 ± 0.06 in the right paw.

2.8. Cold allodynia

Ice-cold water (4 ± 1 °C) was taken in a beaker. The paws of rats with crush injury-induced control and test groups were submerged gently in water and the withdrawal time (s) measured, just prior to (0 h) and at 1, 3, 5 and 7 h after drug administration. The change in withdrawal latency of treated group and vehicle-treated control groups was compared. Results are expressed as mean time in s ± S.E.M.

2.9. Statistical analysis

Data were analyzed by one way ANOVA, followed by Dunnet '*t*' test. **P* values < 0.05 were considered significant.

3. Results

3.1. Behavioural observations

There was no significant difference between right and left paw withdrawal latencies in each group before surgery. At day 1–2 after surgery, the operated leg was flexed back, while sham operated was found normal. At day 7 after surgery, the weight-bearing of the affected leg was reduced, whereas sham operated paw was found normal. After induction of crush injury-induced neuropathic pain,

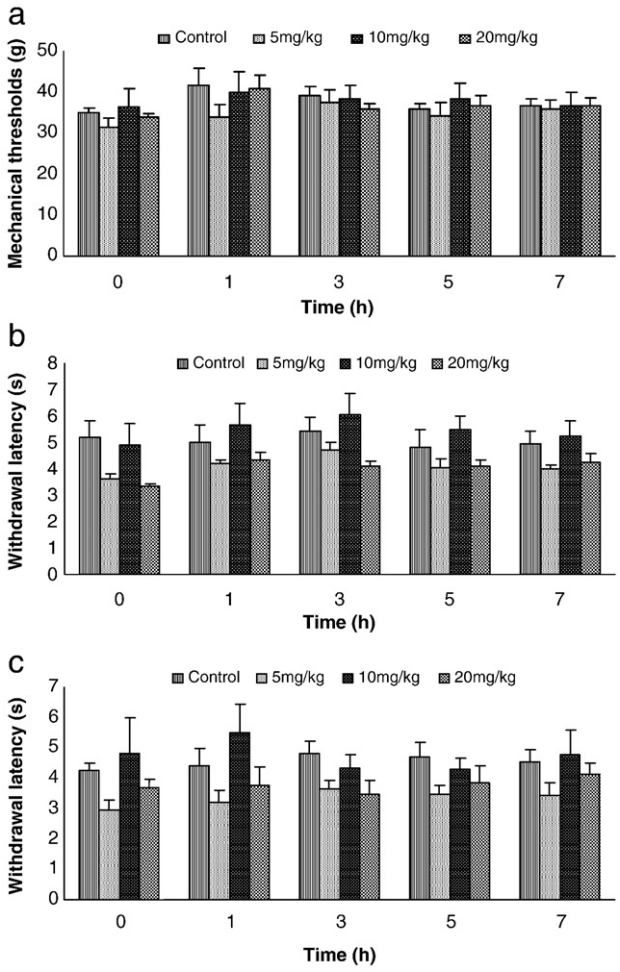

Fig. 2. Effect of intraperitoneally administered ivermectin on crush injury-induced neuropathic pain in hind limb of rats assessed by (a) mechanical stimulation, (b) radiant heat stimulation and (c) cold allodynia. Reaction time was recorded at different time intervals after i.p. administration of drug. The vertical lines at the top of bars represents the S.E.M., $n = 6$.

there were differences among right and left paw withdrawal latencies. Drug treatments did not change the pain threshold in un-injured paw (left).

3.2. Effect of ivermectin on crush injury-induced neuropathic pain in rats

There was no significant effect of ivermectin (5, 10 and 20 mg/kg i.p. or 50, 100 and 200 μg/kg i.c.v.) on pain threshold of crush injury-induced neuropathic rats assessed by mechanical, radiant heat and cold stimulation at 1, 3, 5 and 7 h of drug administration (Figs. 2 and 3).

3.3. Effect of NPPB on crush injury-induced neuropathic pain in rats

3.3.1. Mechanical stimulation

NPPB, at doses of 10, 20 and 40 mg/kg i.p. increased mechanical hyperalgesia in neuropathic rats from 1 to 5 h of observation (Fig. 4a). There was, however, no significant effect of 10 mg/kg of NPPB on thermal hyperalgesia at 3 and 5 h after treatment.

3.3.2. Radiant heat stimulation

In radiant heat stimulation, there was no significant effect of 10 mg/kg at any time point post-drug administration. However, decrease in pain threshold was observed only at 1 h with 20 and 40 mg/kg doses (Fig. 4b).

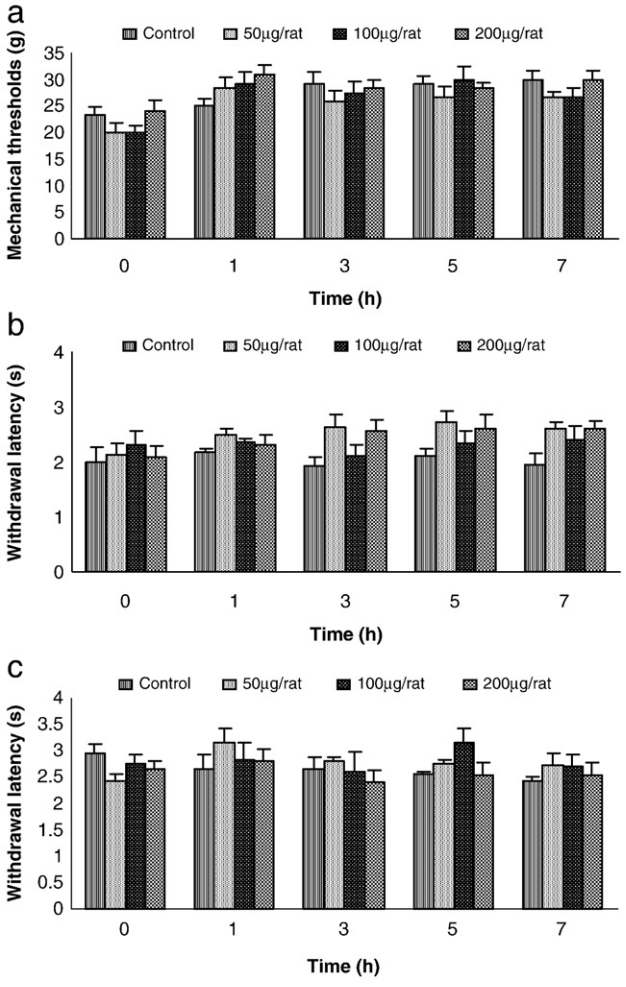

Fig. 3. Effect of intracerebroventricularly administered ivermectin on crush injury-induced neuropathic pain of hind limb in rats assessed by (a) mechanical stimulation, (b) radiant heat stimulation and (c) cold allodynia. Reaction time was recorded at different time intervals after i.p. administration of drug. The vertical lines at the top of bars represents the S.E.M., $n=6$.

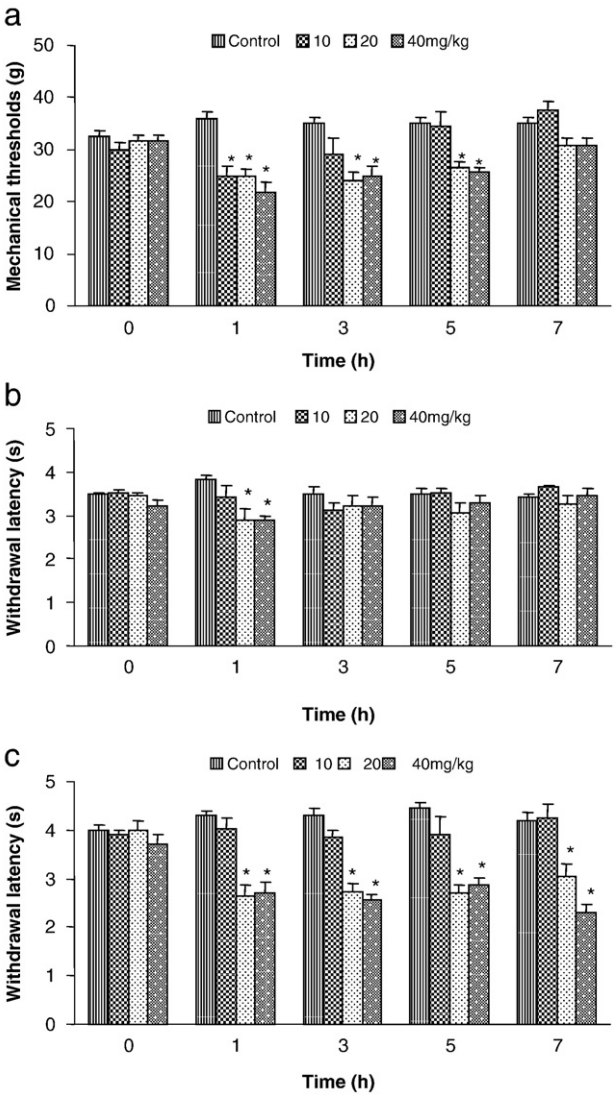

Fig. 4. Effect of intraperitoneally administered 5-nitro-2,(3-phenylpropylamino)-benzoic acid (NPPB), on crush injury-induced neuropathic pain in hind limb of rats assessed by (a) mechanical stimulation, (b) radiant heat stimulation and (c) cold allodynia. Reaction time was recorded at different time intervals after i.p. administration of drug. The vertical lines at the top of bars represents the S.E.M., $n=6$, $^*p<0.05$ compared with control (ANOVA).

3.3.3. Cold stimulation

NPPB, at 20 and 40 mg/kg doses decreased the threshold to pain by cold stimulation from 1 to 7 h of its administration. However, 10 mg/kg dose did not elicit any significant effect on pain threshold (Fig. 4c).

4. Discussion

We attempted to investigate the effect of chloride channel modulators in crush injury-induced neuropathic pain in rats. This is probably, the first study to investigate the effect of ivermectin, a ligand gated chloride channel opener and NPPB, a chloride channel blocker on crush injury-induced neuropathic pain.

Several models of painful neuropathy have been developed to study the mechanism and development of allodynia, hyperalgesia and to assess the effect of various treatments (Kim and Chung, 1992; Kim et al., 1997). Sciatic nerve crush method has been employed by various workers to produce neuropathic pain (Attal et al., 1994; Przewłocki et al., 1999; Bester et al., 2000). After sciatic nerve crush, neuropathic pain symptoms comparable to those of chronic constriction injury model

develop in rats. One of these symptoms is an exaggerated response to light mechanical stimuli referred as mechanical allodynia (Decosterd et al., 2002, 2004). The other symptoms in rats included hyperalgesia, spontaneous pain, abnormal gait and posture of ipsilateral side of sciatic nerve crush at day 3 after injury. The rats could not put weight on affected side.

Our findings with ivermectin at 5, 10 and 20 mg/kg i.p. in crush injury model indicated no significant effect on hyperalgesia throughout the observation period of 1, 3, 5 and 7 h of drug administration. The antiparasitic action of ivermectin is believed to be through a sensitive glutamate-gated Cl^- channel receptor (Glu Cl R) that exists in a number of invertebrate phyla. However, Glu Cl R has not been demonstrated in the vertebrates (Shan et al., 2001). Ivermectin though highly lipophilic substance, is reported not to cross the blood brain barrier (Schaeffer and Haines, 1989), thus we could not find any significant effect on hyperalgesia following i.p. administration. This prompted us to administer it by i.c.v. route at 50, 100 and 200 μg/rat in crush injury-induced neuropathic rats. Ivermectin did not produce any significant effect on hyperalgesia throughout the experiment following i.c.v. administration. Antiparasitic action of ivermectin has also been suggested to be due to its binding with GABA-gated chloride channel thereby, causing hyperpolarization, paralysis, starvation and death of worms but, GABA-gated chloride channels are located centrally in mammals (Schaeffer and Haines, 1989). In an earlier investigation, the action of avermectin (AVM), a family of antiparasitic drugs whose analogues include Ivermectin, selamectin, doramectin and abamectin containing macrocyclic lactone, produced by soil micro-organisms, *Streptomyces avermintilis* was studied in rat cultured hippocampal neurons with patch-clamp techniques. The current activated by AVM was carried predominantly by Cl^- ions, as demonstrated by ion-substitution experiments. The Cl^- channel blocker, picrotoxin (100 μM) substantially but, transiently reduced the AVM response. They also concluded that AVM directly activated Cl^- channels in mammalian central neurons, which resemble the channels activated by physiological transmitters GABA and glycine (Schonrock and Bormann, 1993). Our findings, however, suggested that ivermectin administered by i.c.v. route did not show any significant effect in pain protection in crush injury-induced neuropathic pain. Highly lipophilic substance ivermectin had been found to show unexpectedly poor penetration of blood brain barrier and is now believed that this phenomenon is as a result of the actions of drug efflux transporters (Ayrton and Morgan, 2001). The lack of effect of ivermectin following i.c.v. administration may possibly be due to the presence of *p*-glycoprotein and range of drug transporters such as, MDR, MRP and OATP (Cordon-Cardo et al., 1989; Huai-Yun et al., 1998). Amongst the drug transporters, *p*-glycoproteins have been most widely studied and are expressed in the apical membrane of the brain capillary epithelial cells and are oriented to pump drugs/noxious substrates from inside the cells and back into the blood (Edwards, 2003). Fluorescent-labeled ivermectin (BOD-IPY-ivermectin) is extruded out of brain capillaries by a concentrative mechanism and this export process is reduced by substrate of *p*-glycoprotein without any change in drug uptake (Nobmann et al., 2001). It is therefore, suggested that Ivermectin might have been extruded out even after i.c.v. administration through *p*-glycoprotein and failed to modify crush injury-induced neuropathic pain in this study.

Experiments with NPPB in crush injury-induced neuropathic rats revealed highly significant hyperalgesic effect by decreasing pain threshold at 20 and 40 mg/kg i.p. doses, however, a non-significant effect was observed with 10 mg/kg dose at 3, 5 and 7 h of drug administration, when compared with respective controls. NPPB was found to block CaCCs in bovine pulmonary artery endothelial cells (Nilius et al., 1997), rabbit pulmonary artery smooth muscle cells (Piper et al., 2002) and DRG neurons (Currie et al., 1995). Further, NPPB is quite effective as a blocker of CaCCs as its Ki value is in μM range (Wu and Hamill, 1992). CaCCs have been identified in number of peripheral and central population of neurons. They open in response to increase in $[Ca^{2+}]_i$, that follows Ca^{2+} influx during electrical, sensory or chemical stimuli (Frings et al., 2000).

NPPB is a CaCCs blocker (Currie et al., 1995) and it is expected that it should produce anthyperalgesic effect in crush injury-induced neuropathic pain as has been suggested by other workers (André et al., 2003; Hilaire et al., 2005). These studies were based on axotomy involving patch-clamp studies and in fact, no *per se* effect of CaCCs blockers was studied in animals. Our findings of hyperalgesia following NPPB administration are further supported by a related study where, i.t.-pre-treatment with the Ca^{2+}-activated Cl^- channel blockers attenuated the antinociception induced by i.t. treatment with δ-, but not μ- and κ-opioid receptor agonist in the tail flick test in mouse (Yamazaki et al., 2000). It could be argued that NPPB produces hyperalgesia through some unknown action independent of CaCCs blockade such as, blockade of Ca^{2+}-activated K^+ channels. NPPB was found to inhibit Ca^{2+}-activated K^+ current in glioblastoma cells (Fiorettia et al., 2004). Several Cl^- channel blockers including fenamates, A-9-C and NPPB (10 μM) inhibited Ca^{2+}-activated K^+ current in isolated cells from rat portal vein (Kirkup et al., 1996). Ca^{2+}-activated K^+ channels openers stabilize the cells by increasing efflux of K^+ ion, leading to hyperpolarization and thus, decrease cell excitability and blockade of Ca^{2+}-activated K^+ channel increases neuronal excitability (Ghatta et al., 2006). NPPB-induced hyperalgesia is further supported by an earlier study where stimulation of small conductance Ca^{2+}-activated K^+ channels in sensory pathways including nociceptive processes can be substantially reduced and its blockade by UCL 1848 increases the sensory input i.e. mechanical and heat hyperalgesia (Bahia et al., 2005).

Our results showed that NPPB increased significantly mechanical and thermal hyperalgesia in crush injury-induced neuropathic pain rat model, whereas ivermectin, either by i.p. or i.c.v. route of administration, has no effect on pain symptoms in this model. NPPB hyperalgesic effect is independent of CaCCs inhibition and may be due to blockade of Ca^{2+}-activated K^+ channel.

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