



ORIGINAL ARTICLE

The Changes of GABA transporters (GAT-1 and GAT-3) and GABAA Receptor $\alpha 1$ subunit Expression in the Spinal Cord after Peripheral Nerve injury: Effect of GABAA Receptor Stimulation and Glial Inhibition

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ABSTRACT

Regarding the loss of spinal GABAergic inhibition in neuropathic state, we assayed for protein level of the GABA transporters (GAT-1 and GAT-3) and GABAA receptor $\alpha 1$ subunit in male wistar rats with chronic constriction injury (CCI) model of neuropathic pain (Bennett method, 1988). We also examined whether the GABAA receptor agonist muscimol and glial inhibitor pentoxifylline would modify behavioral tests and also could modulate the level of GABA transporters. Behavioral tests (plantar test and Von Frey) were performed one day before surgery and then on days 1, 4, 7 and 14 after surgery in sham and CCI groups. In group that received muscimol (2 mg/kg) on day 14 after CCI, behavioral tests were examined 30 minutes after drug administration on the same day. Pentoxifylline (30 mg/kg daily) was administered one day before neuropathy and then daily to 14 days after CCI. Behavioral tests were performed 30 minutes after pentoxifylline administration only on day 14. GABA transporters and $\alpha 1$ subunit of GABAA receptor expression were detected by Western blotting. CCI was associated with a considerable reduction in GAT-1 and GAT-3 protein level in the lumbar spinal cord of CCI animals but the level of GABAA receptor $\alpha 1$ subunit did not change in CCI group compared to sham group. Both muscimol and pentoxifylline could reduce hyperalgesia and allodynia but could not modulate the level of GABA transporters. The results showed the loss of GABAergic inhibitory tone in neuropathic state and involvement of GAT-1 and GAT-3 in neuropathic pain development. Glial inhibition and GABAA receptor stimulation were effective in alleviating pain but could not be effective on transporters.

Keywords : CCI, GABA transporters, GABAA receptor, muscimol, pentoxifylline

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INTRODUCTION

The mechanisms underlying neuropathic pain are intricate and controversial. One of the most important mechanism is loss of inhibition in the superficial laminae of the spinal dorsal horn that significantly contribute to the development of neuropathic pain [1]. GABA, a principal inhibitory neurotransmitter of the central nervous system, is concentrated in this region [2]. In the spinal cord, GABA inhibits afferent transmission of nociceptive information through GABAA and GABAB receptors [3]. GABAA receptors are found on the terminals of A δ and C fibers within the spinal cord dorsal horn [4] and stimulation of these receptors can reduce hyperalgesia [5]. Several studies propose that peripheral nerve injury induce a functional loss of GABAergic transmission in the superficial dorsal horn [6, 7]. Muscimol, a GABAA receptor agonist, increases nociceptive threshold in a variety of pain models, including tail flick [8], hot plate and formalin tests [3]. Spinal administration of the GABAA receptor agonists, muscimol and isoguvacine, also attenuate allodynia and hyperalgesia after neuropathy [9]. In rats with spared nerve injury (SNI) model of neuropathic pain, thermal hyperalgesia and mechanical allodynia were attenuated after administration of muscimol [10]. On the other hand, some studies indicated no significant loss of GABA receptors, spinal GABAergic interneurons and its synthesizing enzymes in neuropathic animals

[11]. It has been shown that no changes in the expression of GABAA receptor alpha1 subunit was detected in the spinal cord of CCI rats compared to sham group 4h following ligation of the sciatic nerve [12]. It has been also reported no changes in the GABAA receptor level in rats with SNI model of neuropathy [1] and in partial injury of rat tail innervating nerves [11]. Furthermore, there is evidence of increase, rather than decrease, in the content of GABA in dorsal horn or in GABAergic inhibitory activity of spinal cord following peripheral nerve injury [8]. There are reports that GABA become depolarizing and, thereby, substantially increase the excitability of the cells after neuropathic pain [12, 13]. It is uncertain whether the loss of GABA inhibition is due to loss of GABA neurons, receptors, decrease in their activity or depletion of GABA through the loss of GABA synthesizing enzymes. GABA loss may also result from down regulation and incapability of the GABA transporter proteins (GATs) to recapture and recycle this neurotransmitter, leading to depletion of GABA from neurons. The inhibitory action of GABA is terminated by the uptake of GABA into neurons and glial cells by GABA transporters [8, 11, 14]. GABA transporters are the key mechanism for clearance and maintenance of homeostasis in extracellular GABA concentrations at inhibitory synapses and thus control the termination of signaling at these sites. They regulate GABA content below neurotoxic level and are involved in modulation of magnitude and duration of GABAergic inhibition. So far, four GABA transporters have been cloned and characterized, i.e., (GAT 1-4) [4, 15]. GAT-1 and GAT-3 are exclusively expressed in the CNS and the most probable candidates for regulating GABA concentration in the nervous system, are these two transporters [16]. GAT-1 appears to be the most predominant GAT and is preferentially expressed on neuronal elements although it is also present in glial cells [15, 17]. This transporter is found on presynaptic as well as post synaptic GABAergic [7] and even on non-GABAergic neurons [7, 15]. GAT-3 is primarily expressed in glial cells [18]. Recently, knockdown of GAT-1 was revealed to induce decreased nociceptive responses to various painful stimuli in mice [19], whereas overexpression of GAT-1 and GAT-3 were reported with significant hyperalgesia in inflammatory state [20]. Moreover GAT-1 blocker NO-711 treatment significantly reduced mechanical and thermal allodynia in neuropathic pain [7]. Some studies reported no evidence for loss of GABAergic neurons within dorsal horn of spinal cord in the chronic constriction injury (CCI) [21] and spared nerve injury (SNI) models [22] of neuropathic pain. It is likely that the change in expression of GABA transporters is correlated to nerve injury induced loss of GABAergic activity. Little is known regarding the expression of GAT-1 and GAT-3 in neuropathic pain, and more rigorous studies are needed. Like neurons, glial cells contribute to GABA uptake to control the concentrations of GABA in extracellular space [23]. Recently, numerous studies demonstrate that astrocytes and microglia activation play an important role in initiation and maintenance of neuropathic pain [24, 25]. Thus, suppression of glial activation, which in turn, inhibits the synthesis of proinflammatory cytokines, can alleviate neuropathic pain. Pentoxifylline is a general anti-inflammatory agent that reduces the synthesis of proinflammatory cytokines and inhibits phosphodiesterase activity [26]. Additionally, Pentoxifylline inhibits spinal glial activation [26, 27]. In a rat model of neuropathic pain induced by L5 spinal nerve transection, systemic administration of Pentoxifylline attenuates the development of hyperalgesia and allodynia [27]. It is believed that GAT-1 content is significantly modulated by activity at the GABA receptor [28]. Recent reports also indicate that, the loss of GABAergic tone after spinal cord injury (SCI), may be mediated through glial activation. These reports suggest that glial activation may induce a decrease in spinal GABAergic system inhibitory tone by down regulation of glutamic acid decarboxylase (GABA synthase enzyme). They also showed propentophylline (a potent inhibitor of glial activation) administration for 7 days after SCI, reduced astrocytic and microglia activation and prohibited the loss of GABAergic inhibitory tone by preserving the level of GABA synthase enzyme [29]. In the present study, we induced CCI model of peripheral neuropathy in order to investigate spinal cord expression of GAT-1 and GAT-3 and their relation with pain behaviors. Given the importance of GABA inhibition to processing of sensory information in the spinal cord, we also assayed for protein level of the GABAA receptor α 1 subunit. Moreover we wanted to examine whether systemic administration of muscimol and also preemptive and repeated administration of pentoxifylline could influence thermal hyperalgesia and mechanical allodynia in the CCI model of neuropathic pain. We assumed that CCI could be accompanied by modified levels of GABA transporters (GAT-1 and GAT-3) and it was interesting to investigate whether pentoxifylline and muscimol may prevent the loss of GABAergic inhibitory tone through modulation of GABA transporters expression following CCI.

MATERIALS AND METHODS

Animals

Adult male wistar rats (Pasture institute, Tehran, Iran) weighing 200-250 g at the beginning of the study were used. Animals were housed two or three per cage under standardized conditions in a climate control room with free access to standard food and tap water in a light-dark cycle of 12 h. All efforts

were made to minimize animal suffering and to use the fewest animals needed for experiments . All experimental procedures followed the recommendations of IASP [30] and were reviewed and also approved by the research and ethics committee of Shahid Beheshti University of Medical Sciences .

Neuropathic pain induction

Chronic constriction injury (CCI) of the sciatic nerve was used to induce neuropathic pain according to Bennett and Xie [31]. Briefly , animals were anesthetized with ketamine 60 mg/kg and xylazine 10 mg/kg intraperitoneally . The right common sciatic nerve was exposed at the mid-thigh level by incision through biceps femoris using aseptic surgical techniques. The nerve was freed of surrounding tissue along approximately 1 cm , proximal to the trifurcation of right sciatic nerve and four 4-0 chromic gut ligatures were tied loosely around it , distal to the sciatic notch , with a 1 mm interval between each , until a brief twitch in the relevant hind limb was observed . After checking hemostasis , the wound was irrigated with saline , muscle and overlying skin were closed routinely using 4-0 silk thread and rats were left to recover in heated cages . The rats were observed daily for behavioral signs indicative of neuropathic pain , including signs of autotomy of the injured hindpaw .All surgical procedures were performed by the same person . In sham operated animals , the right sciatic nerve was only freed from surrounding connective tissue but was not ligated .

Nociceptive behavioral testing

Animals were habituated to the testing environment for 3 days before behavioral tests . The threshold responses to painful thermal and mechanical stimuli were assessed by observing paw withdrawal to applied stimuli using plantar test and Von Frey test , respectively . Movements of paw associated with locomotion or weight-shifting were not included as a response . All behavioral tests were carried out in a quiet temperature controlled room between 8:00 AM and 14:00 PM by a trained person and the same person always performed a given test to reduce variability .

Assessment of thermal hyperalgesia

Sensitivity to noxious heat stimulation was evaluated by means of plantar test (Ugo Basile , Italy) . On each testing day , animals were moved in the testing room , were placed in plexiglas chambers with an elevated glass floor and left to acclimate for 30 minutes before testing . A high intensity movable radiant heat source was positioned from below to the mid-plantar surface of the injured hindpaw (right hindpaw) and was controlled by a timer . When an animal withdrew its hindpaw, the heat source was immediately shut off and the timer was stopped . The latency between stimulus onset and paw withdrawal was recorded as paw withdrawal latency (PWL) . Each foot was stimulated three times with an inter-stimulus time of at least five minutes and the average of all three latencies was determined and recorded . A cut-off time was set at 33 s , to prevent tissue damage in the absence of paw withdrawal .

Assessment of mechanical allodynia

In order to evaluate changes in mechanical withdrawal thresholds , the Von Frey test was used . Animals were placed singly in a transparent Plexiglas box on a metal-mesh floor allowing access to the plantar surface of hindpaw and were permitted to acclimate 30 minutes before testing began . Mechanical hypersensitivity of the ipsilateral hindpaw (injured hindpaw) was determined by measuring paw withdrawal threshold (PWT) to tactile stimuli with Von Frey monofilaments of logarithmically increasing stiffness (2, 4, 6, 8, 15, 26 and 60 g , Stoelting , USA) . The calibrated nylon monofilaments were applied from below through the mesh onto the mid-plantar surface of injured hindpaw until slight bending of filaments was obtained . Application of the Von Frey monofilaments always occurred three times at interstimulus intervals of 5 min with approximately 1 s holding in each application for each consecutive filament , starting with the lowest filament . Lifting or flinching of the stimulated paw was recorded as a positive response . The softest Von Frey monofilament that produced a brisk withdrawal or flinching of two out of three applications , was considered as the paw withdrawal threshold (g) . In the absence of a response , the cut-off point was 60 g to avoid tissue damage .

Experimental groups and drug treatment

Rats were randomly assigned to five groups (each group consisted of eight animals) including sham , CCI , CCI + muscimol , CCI +pentoxifylline and CCI + vehicle groups . In sham and CCI groups , thermal hyperalgesia and mechanical allodynia were assessed by plantar and Von Frey tests one day before neuropathy (day -1) and on days 1 , 4 , 7 and 14 after surgery , respectively . In CCI group that received muscimol (2 mg/kg , Tocris , i.p.) on day 14 after CCI , behavioral tests were evaluated on the same day . Pentoxifylline (30 mg/kg , dissolved in 0.9 % saline , Sigma -Aldrich , St. Louis , Mo , USA) was administered intraperitoneally one day before surgery and then daily to day 14 after CCI . Vehicle-treated CCI rats received saline according to the same schedule of pentoxifylline group . In CCI groups that received pentoxifylline and saline , behavioral tests were evaluated only 14 days after CCI . Drugs were injected at least 30 minutes prior to the behavioral tests . In all these groups , after behavioral tests on day 14 after CCI , under anesthesia animals were decapitated , then , the lumbar spinal cords were quickly

removed on iced saline, immediately snapfrozen in liquid nitrogen and stored at -80 °C until GABA transporters and GABAA receptor α 1 subunit expression assessment was conducted by western blotting technique.

Spinal GAT-1, GAT-3 and α 1 subunit of GABAA receptor expression detection by Western blotting

After behavioral tests, Western blot was used to evaluate the variations of GAT-1, GAT-3 and GABAA receptor α 1 subunit expression in the spinal cord. Briefly, all rats were deeply anesthetized, euthanized by decapitation and their lumbar spinal cord was rapidly removed on ice, weighted and homogenized in radio-immuno-precipitation assay (RIPA) buffer containing 50 mM Tris-HCL (pH 7.4), NP40 1%, NaCl 150 mM, 1 mM ethylenediaminetetraacetic acid (EDTA), leupeptin 1 μ l, aprotinin 10 mg/ml and PMSF 2 mM. On 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), equal amounts of samples (50 μ g of total protein per well) were loaded and separated at 100 mv for approximately 1 h. A sample from an individual spinal cord was loaded on each well. Proteins were shifted onto Immobilon-P PVDF membranes (Millipore, Bedford, MA, USA) using the miniprotean II (Bio-Rad) at 110 V for 90 min. The membranes were located in a blocking buffer (0.2% Aurora Blocking Reagent, 1X Phosphate Buffered Saline (PBS): 0.058 M Na₂HPO₄, 0.068 M NaCl, 0.017 M NaH₂PO₄, 0.05% Tween-20 from ICN Biomedicals, Costa Mesa, CA, USA) and nonspecific binding sites on the membrane were blocked by incubation (for 90 min at 24 °C or overnight at 4 °C), thereafter the membranes were incubated (3h, 24°C) with rabbit polyclonal primary antibodies to GAT-1 and GAT-3 (diluted 1:5000, Abcam) and mouse monoclonal primary antibody to GABAA receptor alpha 1 subunit (diluted 1:5000, Abcam). After washing twice with blocking buffer, the membranes were incubated (90 min, 24°C) with secondary antibody in blocking buffer (anti-rabbit for GABA transporters, diluted 1:10000, Abcam and anti-mouse for GABAA receptor alpha 1 subunit, diluted 1:10000, Abcam) for protein detection. The membranes were then rinsed three times with blocking buffer that followed by two immediate washing with assay buffer (20 mM Tris-HCl with pH 9.8, 1 mM MgCl₂). With a chemiluminescence detection system (ECL, Amersham), the immunoreactivity of the proteins on the membranes was detected. The membranes were subsequently incubated in stripping buffer (100 μ M 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl with pH 6.7) at 50°C for 30 min and reprobed with mouse monoclonal β -actin primary antibody (diluted 1:5000, Abcam) to confirm equivalent loading. The protein levels in the spinal dorsal horn were estimated from optical density measurements of scanned images of the respective bands using National Institute of Health (NIH) Image 1.60 software and indicated as the ratio of the intensity of the GAT-1, GAT-3 and GABAA receptor alpha 1 subunit bands to those of β -actin.

Statistical analysis

To compare the results obtained from groups, two-way repeated measures analysis of variance (ANOVA), followed by Bonferroni post-hoc test was used. Unpaired Student's t-test was used to determine significant differences between the groups in Western blot experiments and behavioral experiments. Values were expressed as mean \pm standard error of mean (SEM) and statistical significance was inferred at P-values less than 0.05 ($p < 0.05$).

RESULTS

Development of thermal hyperalgesia and mechanical allodynia in CCI rats

All of the rats that had experienced CCI, often held up the injured hindpaw with the toes plantar-flexed, had a tendency to avoid bearing weight on it and indicated changes in their posture. There were no significant changes of the withdrawal threshold to noxious heat stimulus and the mechanical pain threshold of ipsilateral hindpaw to Von Frey filaments in sham operated rats. Ipsilateral pre-injury (day -1) paw withdrawal latency(s) measured by noxious radiant heat (plantar test) were similar in sham and CCI groups. Thermal hyperalgesia in sciatic nerve ligated rats was monitored as early as post-operative day 4 ($p < 0.01$) and paw withdrawal latency(s) decreased. The values of nerve ligated animals were also significantly lower than those of sham group on days 7 and 14 after neuropathy ($p < 0.001$) (Fig. 1A). Before surgery (day -1) paw withdrawal threshold(g) evaluated by Von Frey filaments (Von Frey test), did not differ between two groups (CCI and sham). All rats with Chronic Constriction Injury exhibited significant mechanical allodynia at the ipsilateral hindpaw, indicating by mean of paw withdrawal threshold(g) to non noxious stimuli (Von Frey monofilaments) significantly decreased compared to the ipsilateral side of sham operated animals on days 7 ($p < 0.05$) and 14 ($p < 0.001$) after CCI (Fig. 1B).

Attenuation of thermal hyperalgesia and mechanical allodynia by muscimol

The effect of intraperitoneally administration of muscimol (2 mg/kg) on thermal hyperalgesia and mechanical allodynia was evaluated by plantar test and Von Frey hairs, respectively, on day 14 after CCI. Treatment with muscimol significantly reduced thermal hyperalgesia (Fig. 2A) and mechanical allodynia (Fig. 2B) on post operative day 14. Therefore rats administered muscimol had a higher paw withdrawal latency ($p < 0.001$) and paw withdrawal threshold ($p < 0.01$) compared to the CCI group.

Attenuation of thermal hyperalgesia and mechanical allodynia by pentoxifylline

The effects of preemptive and repeated systemic administration of pentoxifylline (30 mg/kg) and vehicle (saline) from one day before CCI (day -1) and then daily to post operative day 14, on thermal hyperalgesia and mechanical allodynia were assessed by plantar and Von Frey tests, respectively, in animals with CCI model of neuropathic pain. Pentoxifylline administration suppressed the development of thermal hyperalgesia in CCI animals and significantly increased the PWL in pentoxifylline treated group on day 14 ($p < 0.001$) after surgery when compared to the vehicle group (Fig. 3A). Pentoxifylline administration also alleviated the development of mechanical allodynia in CCI rats (Fig. 3B). Moreover, PWT in group that received pentoxifylline was significantly higher than that in vehicle treated group on day 14 ($p < 0.01$) post surgery.

Quantities of lumbar spinal cord GABA transporters and GABAA receptor alpha 1 subunit after nerve injury and the level of GAT-1 and GAT-3 after GABAA receptor stimulation and glial inhibition

Data were expressed as GAT-1 or GAT-3/ β -actin and GABAA/ β -actin ratios, to normalize differences in protein loading. Protein band densitometry revealed that, loose ligation of sciatic nerve was associated with a considerable reduction in GAT-1 and GAT-3 protein level in the lumbar spinal cord of CCI animals on post-operative day 14 ($p < 0.001$) compared to the sham condition (Fig. 4). We then compared the level of $\alpha 1$ subunit of the GABAA receptor between sham operated and CCI groups 14 days after sciatic ligation. Our data demonstrated that neither the CCI nor the sham surgery elicited significant changes in these levels on post operative day 14 (Fig. 4). We also compared the level of GAT-1 and GAT-3 between muscimol-treated and CCI groups on day 14 after sciatic ligation. Our results showed that, muscimol administration did not alter spinal GAT-1 and GAT-3 expression in CCI rats (Fig. 5). Additionally, we compared the level of GAT-1 and GAT-3 in lumbar enlargement of spinal cord between pentoxifylline-treated and vehicle (saline) groups on day 14 after sciatic ligation. Data analysis indicated that, pentoxifylline administration in the CCI rats did not alter spinal GAT-1 and GAT-3 expression compared to vehicle treated CCI rats (Fig. 6).

DISCUSSION

Here we demonstrated that CCI produced robust thermal hyperalgesia as well as mechanical allodynia and also spontaneous pain. These results are in line with previous studies which CCI model of neuropathic pain was associated with neuropathic pain symptoms [31, 32]. Moreover, we showed down regulation of GABA transporters (GAT-1 and GAT-3) on day 14 after surgical operation but we did not find any changes of spinal GABAA receptor $\alpha 1$ subunit despite the induction of neuropathic pain symptoms after nerve injury. In the present study, muscimol administration reduced thermal hyperalgesia and mechanical allodynia but could not prevent down regulation of GAT-1 and GAT-3 in CCI rats. It has been also indicated that preemptive and repeated administration of pentoxifyllin decreased behavioral responses of neuropathic pain (thermal hyperalgesia and mechanical allodynia) but could not modulate the expression of GABA transporters in rats after CCI.

Previous behavioral studies have demonstrated that administration of GABAA receptor agonists reduced thermal hyperalgesia and mechanical allodynia after peripheral nerve injury [9]. Rode et al. (2005) reported that systemic administration of the GABAA receptor agonist muscimol attenuated both thermal hyperalgesia and mechanical allodynia in rats with spared nerve injury model of neuropathic pain [10]. It has been shown that muscimol administration reduced mechanical allodynia following partial injury of tail-innervating nerves [11]. Gwake et al. (2006) reported that mechanical allodynia is reduced after spinal cord injury in rats administered muscimol [33]. Our data are in line with the above studies. We have shown that muscimol alleviated thermal hyperalgesia and mechanical allodynia in CCI rats. These findings appear to have been mediated by the effect of muscimol on GABAA receptors. On the basis of our results, it is possible that an injury induced reduction in GABAA receptor activity and then low chloride concentration in neurons may contribute to the loss of GABAergic inhibitory system and finally the development of neuropathic pain. Miletic et al. (2008) showed that no changes in the level of GABAA receptor $\alpha 1$ subunit was detected in the spinal cord 4h following loose ligation of the sciatic nerve [12]. Our data are concurrent with the results of Miletic and Miletic, we demonstrated that, muscimol administration could reduce thermal hyperalgesia and mechanical allodynia in CCI rats on post operative day 14 but did not find any significant changes in the GABAA receptor ($\alpha 1$ subunit) expression between sham and CCI groups. Recent studies similarly reported that in the rat tail model of peripheral neuropathy [11] and the SNI model of neuropathy [1], the manifestation of neuropathic pain is not related to the changes in the GABAA receptor level. Therefore, the nerve injury induced loss of spinal GABA inhibition may be related to other GABAergic elements. Loss of the GABAA receptor mediated inhibition in the spinal cord may be due to a decrease in the GABA release from axon terminals of inhibitory interneurons [34]. Some studies have been reported that some neurons in the spinal cord underwent

apoptosis following nerve injury, and proposed that this contributed to loss of GABA and its synthesizing enzyme glutamate decarboxylase [34, 35]. Numerous studies have been shown that another possible mechanism for the loss of inhibition may be down regulation of the potassium-chloride transporter (KCC2) in the spinal cord neurons following nerve injury, therefore in according to this mechanism, GABAA receptor activation may reverse GABA action from inhibition to excitation and contributing to neuropathic symptoms [11, 36]. However, this mechanism can not explain why GABAA receptor agonist muscimol attenuates hyperalgesia and allodynia in CCI animals, implying that GABAergic effect in neuropathic state remains antinociceptive (decreased excitability), but not hyperalgesic (increased excitability). It is also likely that impaired spinal GABAergic inhibition after peripheral nerve injury may be due to the loss of synaptic connections [21].

A number of studies have reported considerable changes in morphology and functions of glial cells in the spinal cord under neuropathic pain conditions [27]. It has been indicated that after peripheral nerve injury, activated astrocytes and microglia play an important role in the development and maintenance of neuropathic pain [24, 25]. In addition, these activated cells can facilitate pain transmission by producing proinflammatory cytokines and other substances [37]. Pentoxifylline, minocycline and propentofylline are the most important compounds that inhibit glial activation and attenuate neuropathic pain by lowering proinflammatory cytokines production [26]. There are many studies that demonstrate, preemptive or post injury administration of glial inhibitors can reduce neuropathic pain symptoms. Pentoxifylline and propentofylline that are general anti-inflammatory agents and inhibit phosphodiesterase activity [26, 29], diminished the development of pain behavior after hind paw formalin injection [27]. Liu *et al.* (2007) have also indicated that in spinal nerve ligation (SNL) model of neuropathic pain, daily administration of pentoxifylline for 7 days, alleviated thermal hyperalgesia and mechanical allodynia [27]. Preemptive administration of pentoxifylline prevents post operative pain in patients [38]. Additionally, Mika *et al.* (2007) showed that administration of pentoxifylline in mice and minocycline in mice and rats for 7 days can decrease neuropathic pain behaviors after peripheral nerve injury [26]. Similarly, administration of minocycline for 10 days attenuated mechanical allodynia in SNL rats [39]. In our study, we found preemptive and repeated administration of pentoxifylline from one day before surgical operation to post operative day 14, could effectively reduce thermal hyperalgesia and mechanical allodynia that is in line with previous studies. All of these studies suggest that glial cells have a critical role in the induction of neuropathic pain, it is possible that systemic administration of pentoxifylline can improve neuropathic pain by inhibition of activated glial cells which are the fundamental source of inflammatory cytokines.

Previous reports suggested that a significant depletion of GABA level in the dorsal horn of spinal cord accompanied nerve injury [6, 7]. One possible mechanism for reduced GABAergic inhibitory tone after nerve injury is that the change in GATs expression is related to impairment of GABAergic activity, which was supported in this study. Our results provided an alternative account for GABA depletion after chronic constriction injury by demonstrating that the content of the GAT-1 and GAT-3 were significantly reduced in the spinal cord of sciatic ligated rats. As it mentioned, GABA transporters have an important role in GABA recapturing into neurons and glial cells, following synaptic activation, and terminate the action of GABA [14]. Reduction in the level of GABA transporters on day 14 after CCI may lead to reduction of GABA content and, thereafter, to the loss of inhibitory action of GABAergic system. Miletic *et al.* (2003) reported that CCI-induced down regulation of GAT-1 and GAT-3 may have contributed to a significant depletion of GABA level within GABAergic terminals in the spinal cord dorsal horn without a concomitant loss of GABAergic inhibitory interneurons [6]. In chronic constriction injured rats, this reduction of GABA content in the spinal cord and then the loss of GABA inhibition, can induce the development of neuropathic pain. It seems that up regulation of GAT-1 and GAT-3 can develop the ability of both interneurons and glial cells for recycling of GABA in the dorsal horn and, ultimately, enhance the effectiveness of inhibitory pathways [6, 28]. On the contrary, down regulation of GABA transporters may decrease recycling of GABA by neuronal and glial elements and result in loss of GABAergic inhibition in the spinal dorsal horn. In the study reported here, reduction in the level of GAT-1 and GAT-3 protein and, indirectly, disinhibition of GABAergic tone, can explain thermal hyperalgesia and mechanical allodynia in animals with sciatic nerve ligation. These data are in accordance with some of the previously published reports that reveal decreased expression of GABA transporters in animal models of neuropathy. Indeed, a decreased expression in GAT-1 was demonstrated in the spinal cord 7 days after nerve injury, and this down regulation was associated with pain hypersensitivity [6, 28]. Other reports suggest that after spinal cord contusion, a significant injury induced down regulation of GABA transporter genes, is seen [40]. All of these previous reports and the present data confirm the importance of inhibitory action of GABAergic system for the processing of sensory information in the dorsal horn of spinal cord. An increased expression of GAT-1 protein was shown by Daemen *et al.* in the spinal cord of CCI animals on day 7 after

loose ligation of sciatic nerve [7]. Gosselin et al. (2010) indicated that in the SNI model of neuropathic pain, an augmented GABA transport was seen through upregulation of GAT-1 protein, in the gracile nucleus, whereas the expression of GAT-3 did not change in this report [41]. In addition, painful facial injections of carrageenan could increase expression of GAT-1 and GAT-3 in the spinal trigeminal nucleus [20]. Lee et al. (2010) reported that, despite the development of mechanical allodynia in a model of peripheral neuropathic pain induced by partial injury of tail-innervating nerves, there was no significant change in the spinal level of these two GABA transporters [11]. Our results are in conflict with these studies and this discrepancy may not only be due to the difference in neuropathy model [11] and severity of thermal or mechanical hypersensitivity induction but may be related to the duration after peripheral nerve injury, because nerve injury induced pain shows early, intermediate and late phases of development, which may be mediated by different mechanisms [12, 36].

Shih et al. (2008) have demonstrated that continual systemic administration of midazolam (a benzodiazepine which act on benzodiazepine binding site of the GABAA receptor) for 7 days is effective in alleviating thermal hyperalgesia and prevent the chronic constriction injury elicited decrease in GAT-1 level in the spinal dorsal horn, suggesting that GAT-1 protein level is significantly controlled by activity at the GABA receptor [28]. In our study, we wanted to assess whether administration of muscimol may prevent down regulation of GABA transporters following CCI. Our results are different from the results of Shih et al. we found that muscimol was ineffective in preventing down regulation of GAT-1 and GAT-3 in CCI rats. This discrepancy regarding the effect of muscimol may be due to the duration of drug administration, as it indicated earlier, for assessment of the expression of GABA transporters, spinal cords were removed in hours after muscimol administration. It seems that muscimol in this short duration that was used, could not modulate the level of GABA transporters and needs longer time period, perhaps days.

Recent studies indicated that glial activation in the SNI neuropathic rats is related to a significant alteration in the spinal cord level of GAT-1 [41]. It is well understood that glial cells modulate and regulate extracellular concentration of GABA by GABA uptake via transporters and are important for synthesis and reuptake of GABA from extracellular space [23]. Any change in the capacity of glial cells to scavenge synaptically released GABA by GABA transporters can modulate the GABA content and GABAergic inhibitory tone [41]. Recent findings reported an important interaction between glial cells and neurons in which activation of astrocytes and microglia contribute to a decrease in spinal GABAergic inhibitory tone [23, 29]. These studies demonstrate that activation of glial cells following spinal cord injury can reduce the inhibitory tone of GABAergic system by down regulation of glutamic acid decarboxylase (GAD), the GABA synthase enzyme, in the spinal dorsal horn. Gwak et al. (2008) demonstrated that administration of propentophylline (a glial and phosphodiesterase inhibitor) in SCI rats, inhibited glial cells in spinal cord, prevented down regulation of GABA synthase enzyme and reduced mechanical allodynia after injury. Additionally, propentophylline prevented the loss of GABA inhibitory tone, as measured by preserved levels of glutamate decarboxylase enzyme, GAD [29]. In our study, we wanted to evaluate whether preemptive and repeated systemic administration of pentoxifylline may prevent the loss of GABAergic inhibitory tone through modulation of GABA transporters expression following CCI. Our data showed that pentoxifylline treatment could not prevent down regulation of GABA transporters and ultimately could not prevent the loss of GABAergic inhibitory tone in CCI rats. As previously mentioned in our results, it seems that pentoxifylline can attenuate thermal hyperalgesia and mechanical allodynia in the CCI model of neuropathic pain via glial cell inhibition and reduction of inflammatory cytokines expression, without involvement with GABAergic system by modulation of GABA transporters expression. Our results are different from the results of Gwak et al. that indicated the loss of GABA inhibitory tone may be mediated through glial activation. This confliction may be related to difference in the neuropathic pain model. However we did not investigate the level of other GABAergic elements such as GABA receptors and its synthesizing enzyme in the CCI model of neuropathic pain and their modulation after glial inhibition.

In conclusion, our evidence support the idea of the loss of GABAergic inhibitory tone in neuropathic states and functional involvement of GAT-1 and GAT-3 in the development of experimental neuropathic pain. Muscimol via activation of GABAA receptors and pentoxifylline by inhibition of glial cells reduced behavioral signs of neuropathic pain, but could not improve GABA transporters level in CCI animals. Therefore it seems that impaired GABAergic inhibition in neuropathic pain states may have a variety of causes.

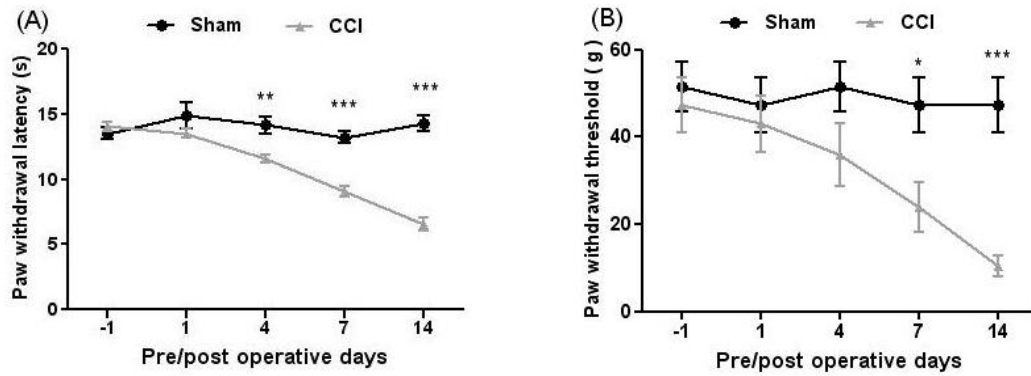


Figure 1: Development of thermal hyperalgesia and mechanical allodynia of the ipsilateral hindpaw of CCI rats . (A) paw withdrawal latency (s) measured by noxious heat stimulus and (B) paw withdrawal threshold (gram) measured by the application of a series of Von Frey filaments , one day before surgery (day -1) and then on days 1 , 4 , 7 and 14 after operation . The data are expressed as mean \pm SEM (n=8) . Inter-group differences were analyzed by ANOVA Bonferroni's multiple comparison tests . * p<0.05 , ** p<0.01 and *** p<0.001.

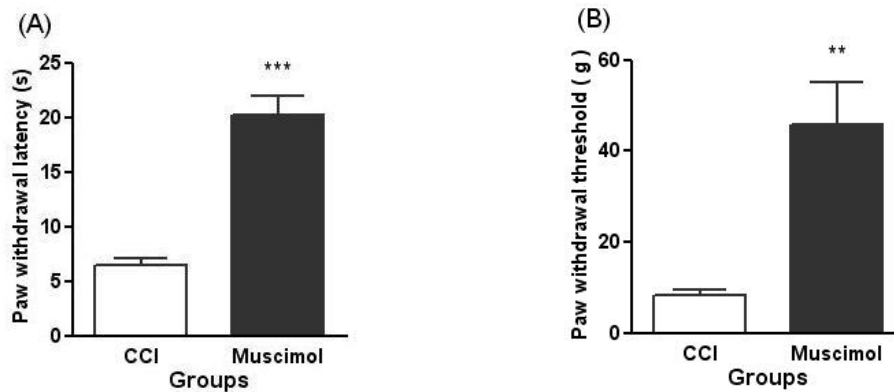


Figure 2: Effect of the GABAA receptor agonist muscimol (2 mg/kg) on the development of (A) thermal hyperalgesia and (B) mechanical allodynia in CCI rats . Hyperalgesia and allodynia were examined at least 30 min after muscimol administration on day 14 after CCI . The data are expressed as mean \pm SEM (n=8) . Statistical significance was assessed by Student's t-test . ** p<0.01 and *** p<0.001.

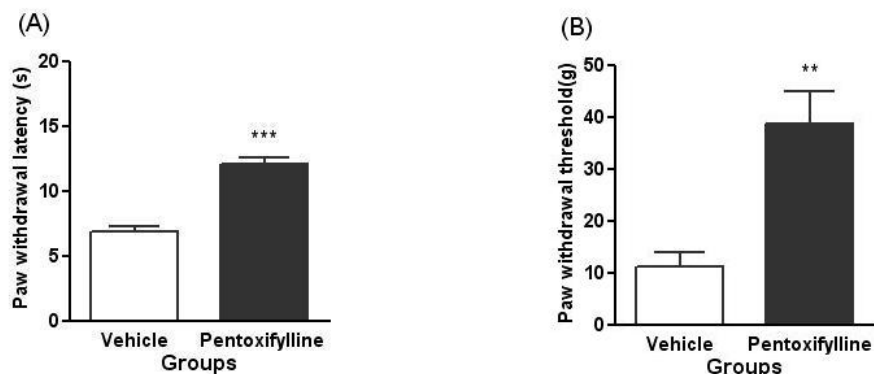


Figure 3: Influence of preemptive and repeated administration of pentoxifylline from one day before surgery (day -1) to post operative day 14 on the development of (A) thermal hyperalgesia and (B) mechanical allodynia after chronic constriction injury in rats . Hyperalgesia and allodynia were evaluated at least 30 min after last doses of pentoxifylline on day 14 after CCI . The data are expressed as mean \pm SEM (n=8) . Statistical significance was assessed by Student's t-test . ** p<0.01 and *** p<0.001.

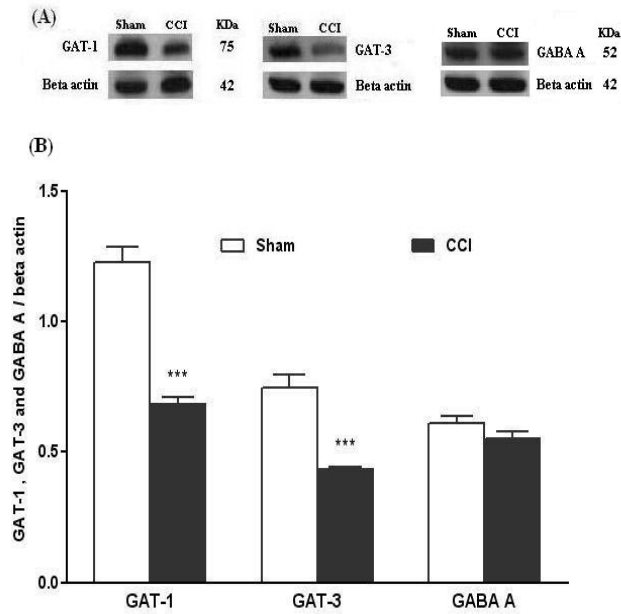


Figure 4: Comparison of the level of GAT-1, GAT-3 and GABA A receptor ($\alpha 1$ subunit) between sham and CCI groups on day 14 after operation. (A) Representative immunoblots of GAT-1, GAT-3 and GABA A receptor alpha1 subunit from sham and CCI rats. (B) Bar histogram showing the band quantification. Note that there is a significant decrease in GAT-1 and GAT-3 protein level in the sciatic ligation animals when compared to sham animals but there is no significant difference in GABA A receptor content in the sham group in comparison to CCI animals. The data are expressed as mean \pm SEM (n=6) and statistical significance was assessed by Student's t-test. *** p<0.001.

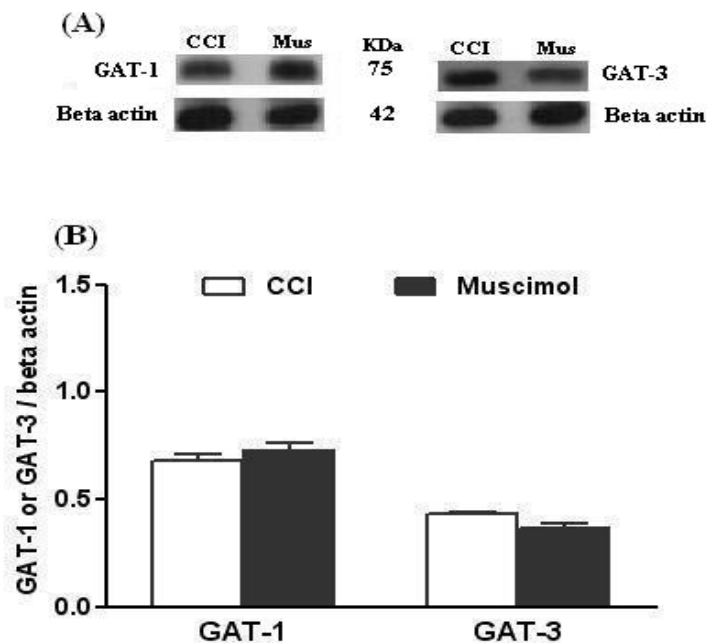


Figure 5: (A) Representative immunoblots of GAT-1 and GAT-3 from muscimol treated and CCI rats. (B) Bar histogram indicating the band quantification. There is no significant difference in GAT-1 protein content and also in GAT-3 content in the muscimol treated animals in comparison to CCI animals. The data are expressed as mean \pm SEM (n=6) and statistical significance was assessed by Student's t-test. Mus : muscimol

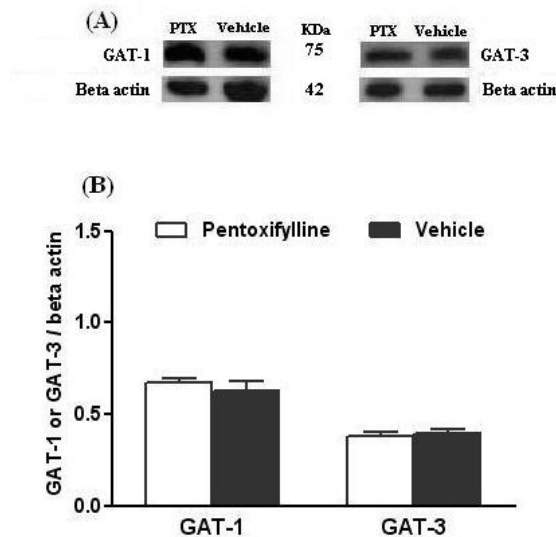


Figure 6: (A) Representative immunoblots of GAT-1 and GAT-3 from pentoxifylline and vehicle treated CCI rats. (B) Bar histogram showing the band quantification. Note that there is no significant difference in GAT-1 protein level and also in GAT-3 level in the pentoxifylline treated animals when compared to vehicle treated animals. The data are expressed as mean \pm SEM (n=6) and statistical significance was assessed by Student's t-test. PTX : pentoxifylline

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