$GABA_A$ receptor plasticity in neuropathic pain: pain and memory effects in adult female rats

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Received 9 May 2018
Accepted 10 February 2019

Egyptian Pharmaceutical Journal 2019, 18:8–15

Background
Neuropathic pain has been shown to increase excitability of neurons. This indicates altered inhibitory mechanism of the nervous system.
Objective
This work was aimed to assess $GABA_A$ receptors plasticity in the brain and spinal cord.
Materials and methods
Fifteen adult female rats were used. Ten animals have their sciatic nerve ligated with no treatment (LIG), and with diazepam treatment for 14 days (LIG+GABA) and the other five were used as the sham group. Pain was assessed using a hot plate and formalin test, while the spatial memory was assessed using Y-maze. At the end of the treatment, the animals were euthanized and fixed using the transcardial perfusion fixation method. The spinal cord, cingulate cortex, and the hippocampus were serially sectioned and stained for $GABA_A$ receptor immunohistochemically. Quantification was done using ImageJ software. Data were analyzed using one-way analysis of variance and Newman Tukey post-hoc test significant level was set at *P* less than 0.05.
Results
A low level of pain was observed in LIG and LIG+GABA animals on both formalin and hot plate test compared with the control. Memory impairment was found only in the LIG+GABA group. Stereology counting showed that $GABA_A$ receptors reduced in the dentate gyrus of the hippocampus of LIG-treated animals which was reversed in LIG+GABA, but in the cingulate cortex, $GABA_A$ receptors were increased in LIG animals and LIG+GABA more than the control while the spinal cord shows no significant difference.
Conclusion
$GABA_A$ agonist treatment did not alleviate the symptoms of neuropathic pain due to GABA signaling changing to excitatory in nature.

Keywords:
cingulate cortex, $GABA_A$ receptor, hippocampus, neuropathic pain, spinal cord

Egypt Pharmaceut J 18:8–15

1687-4315

Introduction

Neuropathic pain (NP) is known to arise due to injury or dysfunction in the peripheral and the central nervous system [1–3]. This dysfunction in the pain pathway leads to hypersensitivity (i.e. the organism feels pain with no stimuli), allodynia (i.e. painless tactile stimulus or warmth elicit pain sensation), and hyperalgesia (i.e. painful stimuli elicits greater intensity of pain sensation) [3]. Sometimes NP is chronic and can results from diabetes, chemotherapy, amputation, and others [4] and it can be induced by chronic constriction injury (CCI) in laboratory animals [5,6] by ligating the spinal cord or peripheral nerve fiber [7]. Using this method, it is discovered that injury to the nerve fibers causes hyper-depolarization in the surrounding spared fiber which are responsible for the chronic pain experienced in NP [8–12]. Another mechanism that has been reported is the disinhibition of the pain pathway and loss of GABA neurons [13,14].

GABA/glycine inhibitory neurons in the dorsal horn are usually activated to reduce pain transmission [3]. With the loss of inhibition, excitotoxicity in NP may lead to the loss of these neurons leading to hyperalgesia [15]. Previous reports have indicated that there are plastic changes in the central nervous system due to CCI [8,16–18]. These changes are responsible for the brain

autoregulatory mechanism to adapt to the injury [19], leading to further propagate the pain experienced [20]. Most of these changes induce the loss of GABA neurons in the spinal cord (dorsal horn) and cingulate cortex (area responsible for sensory processing in rodents) [13,14].

Memory decline is another symptom associated with NP [15,21]. Although the mechanism underlying the decline in memory is not yet understood, some reports have shown that this may be due to excitotoxicity [15]. Mutso *et al.* [22] reported hippocampal-mediated behavioral changes in NP which they associate to ipsilateral upregulation of dpErk molecules.

This study was designed to study whether NP induces $GABA_A$ receptor plasticity in the spinal cord, cingulate cortex, and the hippocampus and activating the receptor will ameliorate the pain and memory deficit in NP.

Materials and methods

Experimental animals

Fifteen adult female rats with an average weight of 150 g were procured from the animal holdings of the Department of Anatomy, Afe Babalola University Ado-Ekiti, Nigeria. The rats were housed in the standard plastic cages of five animals per cage. Food (ret pellets) and water were provided *ad libitum*.

Ethical statement

This experiment was carried out in accordance with the Nigerian National Ethical Code on animal research with formal approval from Afe Babalola University Ethics Committee.

Drug preparation

Commercially available $GABA_A$ receptor agonist (diazepam) injection was purchased from RichyGod International Ltd (Lagos, Nigeria). The drug was prepared into solution by dissolving the ampules in normal saline before administering it to the animals.

Animal grouping

The animals were divided into three groups (sham, ligated, and ligated with treatment of five animals each). Before sciatic nerve ligation, all animals were anesthetized using ketamine Sham (SHAM): animals had their right sciatic nerve exposed and closed up back without ligation. The animals later received 2 ml/kg body weight (BW) normal saline.

Ligated (LIG) group: animals had their right sciatic nerve ligated and later received 2 ml/kg BW normal saline.

Ligated with treatment (LIG+GABA) group: animals had their right sciatic nerve ligated and later treated with 10 mg/kg BW of $GABA_A$ receptor agonist (diazepam).

All administrations were done intraperitoneally and daily for 14 days.

Sciatic nerve ligation procedure

NP was induced by sciatic nerve ligation using the Bennett and Xie model [7]. The animals were sedated with ketamine (2 ml/kg BW intraperitoneal). The animals were placed in a pronated position when deeply anesthetized and were immobilized to the surgical table with a clip. The skin on the right thigh region was cleaned with a cotton wool soaked in ethanol on the dorsal part. The incision was made in this region to expose the sciatic nerve at the mid-thigh level; the nerve was separated from the surrounding tissues, it was raised up with forceps, and three ligatures were carefully tied around the sciatic nerve with 6-0 silk surgical sutures – VCP 496. P30 [23].

Animals in the sham control group had their thighs opened to expose the sciatic nerve and their thigh sutured back without ligating the sciatic nerve.

After the surgical procedure the incised region was treated with procaine penicillin (antibiotics) to avoid infection. Treatment was started 3 days after the surgical procedure.

Behavioral studies

The animals were exposed to a battery of tests to assess the level of pain and memory due to sciatic nerve ligation and or diazepam treatment.

Hot plate test

The aim of this test was to determine the level of pain sensation in the animals termed thermal hyperalgesia [24]. A transparent box made of Pyrex was placed on the hot plate to prevent the animals from roaming around on the hot plate and the animal was placed within the box on the regulated hot plate set at 55°C. Timing started when the animals were placed in the box. Once the animal starts flicking/licking its paws or tail, the timer was stopped and the time was recorded. After this, the animal is returned back to its home cage.

Formalin test

This involves injecting the paws of the animal with 2% formal saline [25]. The rat was then placed down and the number of times it beats (taps) its leg to the ground

in 1 min is taken and recorded as an acute stage. Twenty minutes later, the same recording was taken again from the animals termed the chronic phase which was centrally mediated.

Y-maze test

The Y-maze was used in assessing the spatial working memory of the rats [26]. The rat was placed facing the edge of the maze and monitored on a screen while being timed for 5 min. Visiting the three different arms consecutively was termed right decision (right) while visiting one arm more than once in three alternations was termed wrong decision (wrong). Memory index was calculated as the percentage of right decisions for each animal.

The behavioral studies were done in the order of Y-maze, hot plate, and formalin tests with a day interval each.

Animal sacrifice

After the last behavioral protocol, the animals were deeply anaesthetized with 10 ml/kg body weight of ketamine intraperitoneally, after which the animals were fixed transcardially by flushing the blood with 0.9% normal saline and later with 10% formal saline solution. The brain and the spinal cord were dissected out and postfixed in 10% formal saline solution.

Detection of $GABA_A$ receptor in the spinal cord, cingulate cortex, and the hippocampus using immunohistochemical staining

Immunohistochemical staining was performed using the heat method of antigen retrieval. Brain and spinal cord slices already processed and paraffin embedded were sectioned serially, placing every 10 sections on the slides. Slides were baked in the oven at 50°C for 30 min Then the slides were placed in xylene for 10 min, rehydrated in 100, 90, 70, and 50% of alcohol for 10 min each and then rinsed with distilled water. The slides were placed in hot citric acid solution (antigen retrieval solution) at a pH of 7.0 heated to 70°C for 40 min till it cools down to room temperature. Then the slides were rinsed with distilled water and 1xPBS thrice for 5 min each.

Tissue area was encircled using a PAP pen and then incubated in H_2O_2 for 30 min at room temperature to block endogenous peroxidase followed by protein block solution for 10 min at room temperature and the slides were rinsed with PBS in between the incubation.

The slides were incubated in rabbit anti-$GABA_A$ receptor polyclonal antibody from Novus Biologicals (1:100 NB100-61096, Novus Biologicals Centennial CO, USA) at room temperature for 180 min. Later they were incubated with goat polyvalent antimouse/rabbit secondary antibody from Abcam (1:100 ab64258, Abcam Cambridge, MA, USA) at room temperature for 60 min. Chromogen development was done in accordance with the manufacturer manual of DAB Substrate kit (ab64238; Abcam Cambridge, MA, USA). The slides were counterstained in 1% aqueous hematoxylin solution from Emsdiasum (26042-1; Emsdiasum Hatfield, PA, USA) for 20 min and dehydrated back in 50, 70, 90, and 100% of alcohol for 10 min each and later were cleared in xylene for 10 min and were covered slipped with DPX and then left to dry and viewed under a microscope (Olympus microscope attached with Winjoe 5MP cameroscope).

Stereology

The region of interest [dorsal horn of the spinal cord, anterior cingulate cortex, and dentate gyrus (DG) of the hippocampus] was viewed under a microscope at x40 objective. Positive cells were counted using ImgaeJ software (ImageJ Wisconsin, USA) and recorded.

Statistical analysis

Data were expressed using mean±SEM and were analyzed using one-way analysis of variance. Newman Tukey post-hoc test was done when the analysis of variance shows significance. The *P* value was set at 0.05. The analysis was done using GraphPad Prism software (San Diego, CA, USA).

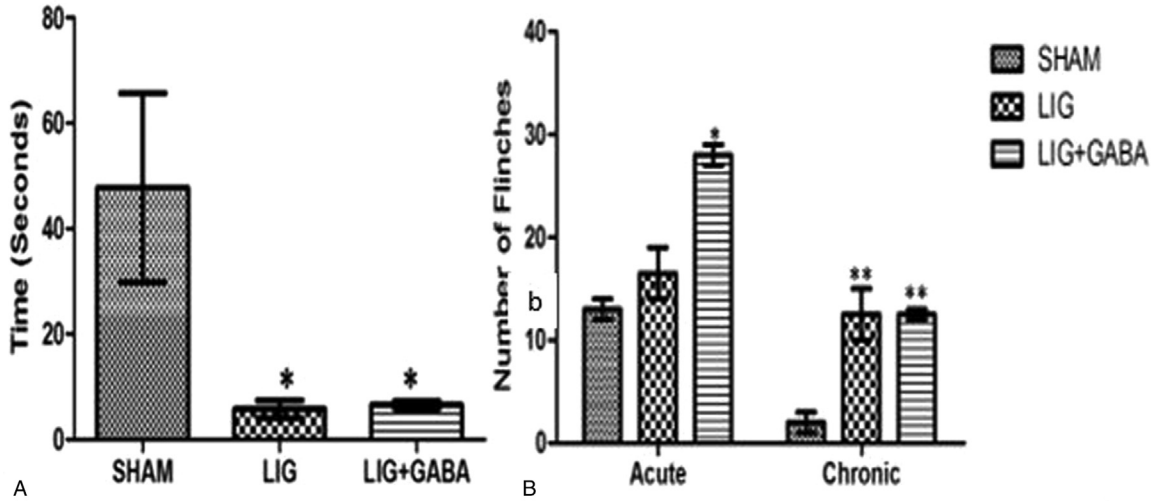
Results

Increased hyperalgesia seen in LIG and LIG+GABA

Diazepam treatment fails to ameliorate pain perception in ligated animals as seen from the hot plate test. Ligated animals (LIG) and animals treated with diazepam (LIG+GABA) showed a significant reduction in the time spent before the experience of pain compared with SHAM animals (Fig. 1a). No significant difference was seen between the GABA-treated and ligated animals.

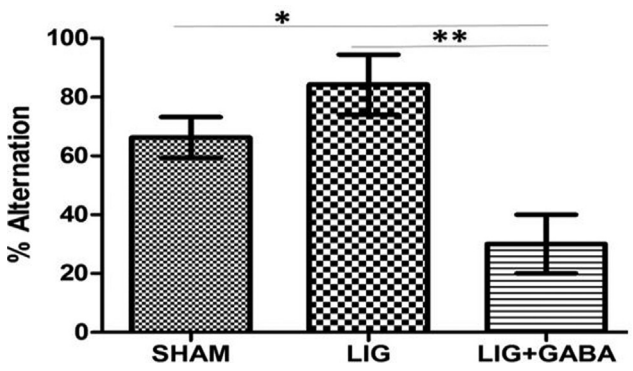
Paw flinching during the formalin test showed that the LIG+GABA-treated group exhibited the highest number of flinches both at an acute and chronic phase which is statistically significant compared with the SHAM group. During the acute phase, no significant difference was observed between the LIG group and SHAM group as this pain is mediated peripherally contrary to the chronic phase which was

Figure 1



The graph showed the results of the pain test. (a) The latency of pain in animals during hot plate test set at 55°C. The ligated animals (LIG, LIG +GABA) had a low threshold of pain as seen from the graph with a significant decrease ($P<0.05$) in the latency of time. Animals treated with GABA agonist after ligation (LIG+GABA) did not show improvement in their threshold of pains compared with the SHAM and no significant difference was observed in the pain threshold of ligated and GABA-treated ligated animals. (b) The pain perception results from formalin test (acute and chronic phase). At acute phase animals treated with GABA agonist (LIG+GABA) showed the highest number of paw flinches which was significantly higher ($P<0.05$) compared with the sham and ligated animals only (SHAM, LIG). No significant difference was observed when SHAM and LIG were compared. Ligated animals and those treated with GABA agonist (LIG, LIG+GABA) have increased the number of flinches, which are significantly higher ($P<0.01$) compared with sham animals. A significant difference was not observed when LIG and LIG+GABA when compared in the chronic phase.

Figure 2



Graph showing the spatial memory index of animals in Y-maze test. No significant difference was observed between the sham animals and the ligated animals (SHAM vs. LIG). Animals treated with GABA agonist after ligation of the sciatic nerve (LIG+GABA) showed a significant reduction in their spatial memory index compared with the sham animals and ligated animals.

mediated centrally where LIG is significantly differenced from the SHAM group but not to the LIG+GABA-treated group (Fig. 1b).

Neuropathic pain and spatial memory

Spatial memory impairment was observed only in the LIG+GABA group which was significantly lower compared with SHAM and LIG groups. Moreover, no significant difference was observed between SHAM and LIG (Fig. 2).

NP effect on hippocampal, cingulate cortex, and spinal cord $GABA_A$ receptor plasticity

$GABA_A$ receptors were expressed at all levels of the spinal cord of experimental animals (Fig. 3a). Stereological counts of positive cells in the spinal cord showed that the sciatic nerve ligation slightly reduced the expression of GABA receptors with further reduction in GABA receptor agonist treatment (Fig. 3b).

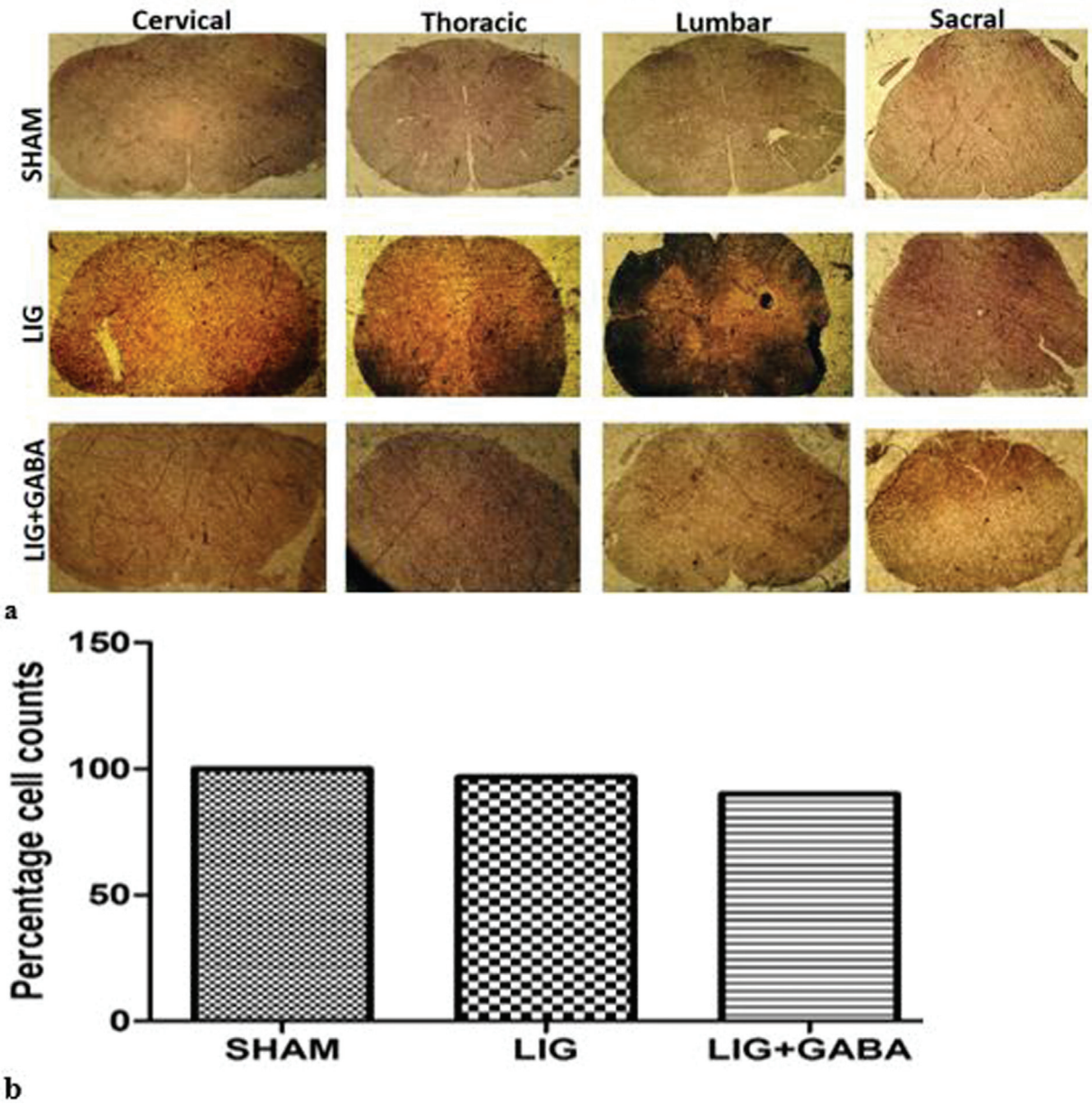
Hippocampal and cingulate serial sections expressed $GABA_A$ receptors (Fig. 4a). Stereology counting showed that the receptors were reduced in the DG of the hippocampus of ligated animals which was reversed by $GABA_A$ receptor agonist treatment (LIG+GABA) more than the control (Fig. 4b). Cingulate cortex (Cg) which processes sensory inputs in rodents expressed more GABA receptors in ligated animals and further increased by $GABA_A$ receptor agonist treatment (LIG, LIG+GABA) more than the control (Fig. 4b).

Discussion

Pain perception

In this study, formalin and hot plate test were used to monitor the pain response in the animals. Hot plate and formalin test showed that the ligated animals (animals induced with NP) showed hyperalgesia because they responded to the stimulus faster than control animals (sham), and the treatment with $GABA_A$ receptor agonist (diazepam) did not

Figure 3

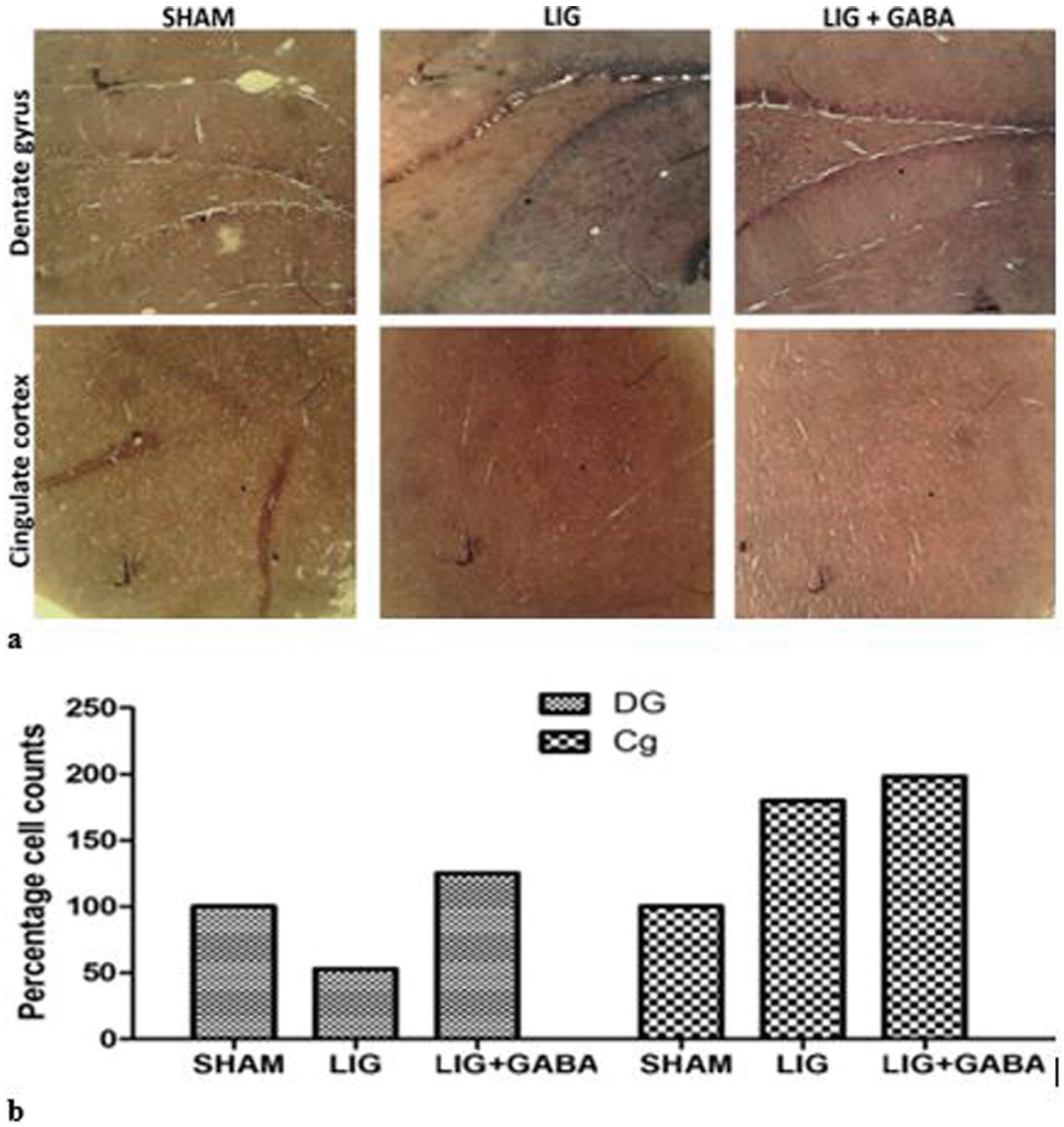


GABA receptor plasticity in the spinal cord at all levels. (a) Immunohistochemical slides of the spinal cord of experimental animals stained for GABA receptors at ×40. GABA receptors were expressed in both anterior and posterior horns of the spinal cord at all levels. (b) GABA receptor immunopositive cell counts in the posterior horn of the spinal cord of experimental animals. There was a steady decline in the number of expression in the ligated animals and those treated with GABA receptor agonist (LIG, LIG+GABA) compared with the control.

alleviate hyperalgesia in the animals. Previous studies have shown that the sciatic nerve ligation model of NP induced loss of activity on the injured nerve and hyperexcitation of the surrounding nerve fibers [15,27]. Hyperexcitation of the nerve fibers has been involved in the pathophysiology of NP [27–29]. It has been suggested that this is due to the system plasticity to correct for the activity of the injured nerve [19]. In a report by Abdulmajeed *et al.* [15], peripheral and center nerve damage was reported, which was associated with excitotoxicity due to sciatic nerve ligation of NP.

GABA neurons in the spinal cord are inhibitory in nature; they regulated the level of pain transmission through the spinal cord. Activating $GABA_A$ receptors (using diazepam) failed to improve pain perception as it further increased pain perception in this model of NP. This was similar to what was reported by Ran *et al.* [28], who showed that muscimol topically injected at the site of nerve damage did not alleviate thermal hyperalgesia in NP rats. Although, in a report by Chen *et al.* [30], they showed that single intraperitoneal injection of diazepam at 9 days after CCI alleviates mechanical allodynia and thermal

Figure 4



GABA receptor plasticity in the hippocampus and cingulate cortex. (a) Slides show the dentate gyrus of the hippocampus and cingulate cortex of experimental animals stained for GABA receptors at ×100. GABA receptors were expressed in all regions under view. (b) GABA receptor immunopositive cell counts in the dentate gyrus of the hippocampus (DG) and cingulate cortex (Cg) expressed as a percentage of control. GABA receptor expressing cells were reduced in the dentate gyrus of the hippocampus of ligated animals, whereas it is more in the GABA-treated ligated animals. Cingulate cortex where sensory processing takes place had more GABA receptors expressing cells in ligated and GABA-treated ligated animals compared with control animals.

hyperalgesia in NP rats. This result was not consistent with other reports that showed that glycine which was a major inhibitory neurotransmitter in the spinal cord showed abnormal signaling and reduced expression in the chronic constriction model NP [29].

Another mechanism that explained the activation of $GABA_A$ receptors not alleviating mechanical pain is that they may contribute to enhancing excitation and not inhibition. Ran *et al.* [28] reported that $GABA_A$ receptors of the adjacent intact dorsal root ganglion may be crucial in the development of hyperalgesia in NP. Ford *et al.* [31] reported that KCC2 signaling responsible for polarization for the inhibitory mechanism of GABA was impaired in NP. The authors also showed that administering adenosine receptor agonist did not have any analgesic effect until when KCC2 signaling is restored. This showed that the expression of KCC2 is altered in NP, which may mimic what happens during neurodevelopment

where NKCC1 was expressed more making GABA transmission to be excitatory [32].

Memory

Memory impairment has been associated with the development of NP [21]. The underlying mechanism associated with these has been proposed to be abnormal signal transmission and plastic changes due to the nerve injury [19]. Hippocampus the main structure responsible for memory formation is also reported to be involved in the transition from acute to chronic pain [33]. NP is reported to cause plastic changes in different regions of the brain with hippocampus inclusive [22,33,34]. The present study showed that ligated animals have no memory impairment on Y-maze compared with the control, but diazepam intervention leads to memory decline. Although memory deficit was reported in neuropathic animals no memory deficit was seen on Y-maze test [15].

Activation of $GABA_A$ receptors leads to memory decline in NP in this study. NP has been shown to increase the level of GABA in the hippocampus [33]. Increasing GABA will stimulate the receptors in the brain, additional stimulation of GABA receptors led to hyperstimulation of this receptor which can lead to long-term depression leading to memory loss [35,36].

$GABA_A$ receptor plasticity

NP has been reported to induce plastic changes in the spinal cord [8]. The major prone areas of these changes have been the dorsal horn since it carries the sensory signals to the brain [37]. Spinal cord inhibitory neurons played a regulatory role in dampening the hyperexcitation seen in NP has been shown to be affected by the plastic changes in NP [8,38]. The present study showed that sciatic nerve ligation induced a slight decrease in the number of $GABA_A$ receptor expressing cells, which is further reduced with diazepam treatment. Although GABAergic neurons have been reported to reduce in the dorsal horn of the spinal cord [8,16–18], the receptors for the neurotransmitter released by these neurons also showed signs of reduction in correlation with production reduction. Although there was no significant difference in the loss of $GABA_A$ receptors, this was similar to what was reported by Polgár and Todd [38].

Cingulate cortex which is responsible for sensory processing in rodents [2,39] is also affected by NP [14]. This region is a key component of the pain pathway. Plastic changes in this region have been hypothesized to be a key in the persistent pain experienced in NP [40,41]. The cortical interneuron reorganization has been identified to occur in the cingulate cortex due to NP [14]. Loss of GABA-inhibitory transmission is known to be a key in the pathophysiology of NP [42]. There was an increase in $GABA_A$ receptor expression in NP animals, activation of $GABA_A$ receptor in the NP animals led to a further increased expression of $GABA_A$ receptors. This may be due to the receptor dynamics to mop up the excess agonist from the system. The animals treated with $GABA_A$ receptor agonist still have hyperalgesia. This indicated that increased $GABA_A$ receptor activation was not leading to inhibition but more excitation of pain pathway as it was reported by Blom *et al.* [14], who showed that cingulate cortex disinhibition led to increased symptoms of NP.

$GABA_A$ receptor was reduced in the DG of the hippocampus in neuropathic animals with increased expression in $GABA_A$ receptor agonist-treated animals. The reduced expression of $GABA_A$ receptor in neuropathic animals explained why there was no memory impairment at this stage on Y-maze as the brain may use this mechanism to regulate the activity of GABA which was seen in neuropathic animals reported by Saffapour *et al.* [33].

Increased expression due to agonist treatment can be explained that diazepam treatment initiated the increase in production of the receptors for binding causing increased inhibition leading to memory impairment.

Conclusion

NP induced plastic changes in the expression of $GABA_A$ receptors in the brain and the spinal cord. $GABA_A$ receptor agonist treatment has no ameliorative effect in NP as GABA signaling may have shifted to excitatory in nature.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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