GABA_A receptor plasticity in neuropathic pain: pain and memory effects in adult female rats
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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), alldynia (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

Background
Neuropathic pain has been shown to increase excitability of neurons. This indicates altered inhibitory mechanism of the nervous system.

Objective
This work 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

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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

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 camerscope).

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 ImageJ 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
mediated centrally where LIG is significantly differed 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).

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
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 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 x40. 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.
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.

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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 x100. 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.
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.

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**Conflicts of interest**

There are no conflicts of interest.

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