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RESEARCH

# Is Transauricular Vagus Nerve Stimulation the Spark for Modulation of Synaptic Alterations in Ulcerative Colitis?

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## Abstract

**Objective** The group of diseases that include Crohn's disease and Ulcerative colitis, characterized by chronic inflammation in the gastrointestinal tract, is called inflammatory bowel disease. It still cannot be treated adequately with the treatment methods, including surgery. Our study aimed to investigate the effect of transcutaneous auricular vagal nerve stimulation (tVNS) on inflammation in colonic tissue and synaptic proteins in the enteric nervous system in rats with experimentally induced colitis.

**Methods** In our study, 9 (total 36) male Sprague–Dawley albino (180–230 g) rats representing four groups were used. Groups: The Sham group was determined as the Sham + tVNS group, Colitis group, and Colitis + tVNS group. The Sham group was intracolonic injected with saline. The Colitis group was intracolonic injected with trinitrobenzene sulfonic acid (TNBS). TVNS was performed in animals for 30 min, 2 times daily for ten consecutive days. Enteric nervous system synapse structures were evaluated using the immunohistochemical method.

**Results** As a result of the study, we can say that in general, the number of Neuroligin-1 and Neurexin-2α positive cells and immunoreactivity percentages were significantly decreased in the TNBS-induced colitis groups compared to the sham groups, while they increased in the tVNS-applied groups. The results suggest that tVNS supports neuronal recovery while treating inflammation in the colon.

**Conclusion** Our study is the first in the literature to show the effects of tVNS on ENS synapse formation. With the results we obtained, we can say that tVNS is effective in improving inflammation in the colon tissue and activates enteric neurogenesis by increasing the number and immunoreactivity of Neuroligin 1 and Neurexin 2 positive cells.

**Keywords** Vagus nerve · Transauricular vagal nerve stimulation · Neuroligin-1 · Neurexin-2α · Colitis · TNBS · Rat

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## 1 Introduction

Inflammatory bowel diseases (IBD) are diseases of autoimmune origin, characterized by chronic inflammation of the gastrointestinal tract [1]. The etiology and pathogenesis of IBD still need to be fully discovered despite extensive immunological, biochemical, microbiological, and epidemiological studies [2]. Ulcerative colitis is a chronic, inflammatory, relapsing bowel disease of the colon and rectum and is most common in people in their 30 s and 40 s. Its incidence has increased significantly recently. Immunosuppressive drugs are mainly used in the treatment. Surgical treatment is indicated if symptoms such as resistant toxic megacolon, colon perforation, polyps, dysplasia, carcinoma, and unstoppable bleeding develop during treatment [3]. Although the medical and surgical methods used in the treatment partially cure the patient,

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the risk of disease recurrence is quite common. Therefore, new treatment methods are needed [4].

The vagus nerve, the tenth and longest cranial nerve with somatic afferent distribution to the outer ear, is an essential component of the parasympathetic part of the autonomic nervous system (ANS)[5]. It has a vital role in regulating metabolic homeostasis [6]. A mixed nerve activates the hypothalamic–pituitary–adrenal axis with its afferent branches and activates the cholinergic anti-inflammatory pathway with its efferent branches [7]. Vagus nerve stimulation (VNS) is a technique that manually or electrically stimulates the vagus nerve [8]. American neurologist James Corning was the first to use VNS and introduced it to the world in the late nineteenth century [9]. VNS can affect basic brain functions and the whole organism [10]. In recent years, functional magnetic resonance imaging studies have shown that the vagus nerve affects the central nervous system (CNS) by non-invasive stimulation of the somatic afferent fibers with VNS, and both visceral and somatic fibers are activated [5, 11]. VNS devices for the treatment of refractory epilepsy, chronic treatment-resistant depression, episodic cluster headaches, and migraine have been approved by the US Food and Drug Administration (FDA) [11–13]. VNS can activate innate "protective" mechanisms, such as anti-inflammatory effects, modulation of neurotransmitter release, enhancement of neural plasticity, reduction of blood–brain barrier permeability, promotion of vascular regeneration, inhibition of apoptosis, and autophagy. These multifaceted effects have led to the official approval of VNS for the clinical treatment of various conditions, including epilepsy, depression, headache, stroke, and obesity. Anti-inflammatory treatments have also begun to be used to treat cardiovascular and cerebrovascular diseases [14]. Numerous studies suggest that VNS may exert anti-inflammatory effects via the cholinergic pathway. The cholinergic activity of the efferent branch of the vagus nerve has an immune-inflammatory regulatory effect called the cholinergic anti-inflammatory pathway (CAP). In particular, the efferent branch of the vagus nerve secretes acetylcholine (ACh), which activates α7nAChR receptors on immune cells and initiates the Jak2-STAT3 pathway [15]. This leads to changes in the levels of inflammation-associated cytokines (e.g., TNF-α, IL-6, and IL-1β). Activation of α7nAChR in VNS is necessary for the emergence of anti-inflammatory effects [16]. Studies have shown that VNS has anti-inflammatory effects during gastrointestinal inflammation in rats and improves colitis. A pilot study on Crohn's disease has been demonstrated that VNS restores vagal nerve tone and reduces inflammation in patients [17, 18].Therefore, VNS can be used as a non-pharmacological alternative treatment for diseases such as enteritis, renal inflammation, and rheumatoid arthritis [19].

The ear is the only place where the superficial distribution of the vagus nerve is found [11]. Electrical stimulation of the vagus nerve's auricular branch (aVN) is an emerging technology in bioelectronic medicine. While Eastern medicine has benefited from the analgesic effects of auricular acupuncture for thousands of years [20–22], the concept of electrically stimulating the auricle is more recent [23]. Stimulation of the aVN regulates the parasympathetic activity of the body by inhibiting harmful sympathetic dominant processes, improves peripheral perfusion due to decreased sympathetic activity, and reduces excessive inflammation. Transauricular VNS (tVNS) is considered to have great potential to treat many ANS-related diseases. Increasingly, studies report that tVNS has therapeutic effects on various diseases and can reorganize the homeostatic balance in patients with impaired body functions [5]. The cutaneous neural innervation of the ear is complex, with the inner part of the concha and tragus considered the most suitable sites for vagus nerve stimulation [24]. In our study, the inner region of the tragus was used in rats to apply tVNS.

The gastrointestinal tract has its unique nervous system that operates independently of the CNS. This system is called the Enteric Nervous System (ENS) [25–27]. The ENS, consisting of neurons and glial cells, is located along the wall of the gastrointestinal tract [28, 29]. The ENS consists of two main plexuses, myenteric plexus (Auerbach plexus) and submucosal plexus (Meissner plexus), and some minor plexuses [30, 31]. In general, the role of the myenteric plexus is to coordinate muscle movements that increase nutrient content. The submucosal plexus plays a vital role in secretion and absorption [25].

Neurexins and Neuroligins are synaptic cell adhesion molecules that connect presynaptic and postsynaptic neurons, mediate signaling, and shape the properties of neural networks [32]. Neurexins are located in the presynaptic terminal; Neuroligins are located in the postsynaptic terminal. Neuroligins form transsynaptic complexes with Neurexins [33, 34]. This transsynaptic complex is essential for effective neurotransmission and is involved in the formation of synaptic contacts [35]. These proteins function to induce synapse formation and maturation [36], mediate synaptogenesis by secreting proteins such as pentraxins to transmit signals between neurons [37]. Recent evidence highlights the importance of transsynaptic interaction between presynaptic Neurexins and postsynaptic Neuroligins. These transmembrane molecules bind to each other outside the cell to increase adhesion between dendrites and axons. Remarkably, Neuroligins alone can induce the formation of fully functional presynaptic terminals upon contact with axons. Conversely, Neurexins alone can induce postsynaptic differentiation and recruitment of receptors in dendrites. Therefore, the Neuroligin-Neurexin interaction uniquely acts as a bidirectional trigger of synapse formation [38]. Recent in vivo studies suggest that Neurexins and Neuroligins play

essential roles in the assembly and maturation of molecular components in synapse development [39]. Studies have shown that no single molecule or molecular family is required for synapses to come together and that Neurexins and Neuroligins are the most important of these regulatory molecules. Studies show that Neurexins and Neuroligins have an essential role in synaptic transmission, especially in GABAergic synapses. Still, it is also true that more studies are needed to evaluate the in vivo functions of these complex protein families [40]. Different synapses (excitatory or inhibitory synapses) are formed by different Neuroligin-Neurexin combinations. Neuroligin 1 is usually found in neurons in excitatory glutamatergic postsynaptic regions [41, 42]. A recent study found a link between Neurexins and GABA receptors [43]. It has been demonstrated that mutations in the Neurexin-Neuroligin complex lead to an imbalance of inhibitory and excitatory activity in neuronal circuits that play a role in the pathophysiology of autism spectrum disorder and schizophrenia [44–46].

The anti-inflammatory properties of vagus nerve stimulation have been reported in various experimental models of inflammation. Our aim in this study was to create an experimental colitis model in rats, evaluate presynaptic-postsynaptic protein numbers and immunoreactivity densities immunohistochemically, and investigate the effects of tVNS on these changes.

## 2 Materials and Methods

This research was conducted at the Gazi University Faculty of Medicine Experimental and Clinical Research Center (GUDAM) between June 2021 and August 2021. This study was approved by the Gazi University Animal Experiments Local Ethics Committee (Approval No: G.Ü.ET-21.041). The study received support from the Gazi University Scientific Research Projects Unit under project number TTU-2021–7301.

### 2.1 Groups

Male Sprague–Dawley albino rats weighing between 180–230 g were used in the research. The rats were randomly divided into four main groups consisting of 9 rats (Table 1). Group 1 (n = 9 Sham group) was given physiological saline intracolonically after anesthesia. Then, the clamps used in tVNS were placed on their ears, and no stimulation was given for 30 min. Group 2 (n = 9 Sham + tVNS group) was given physiological saline intracolonically after anesthesia. Then, the clamps used in tVNS were placed, and their ears were stimulated for 30 min. Intracolonic TNBS was applied to Group 3 (n = 9 Colitis group) after anesthesia. The clamps used in tVNS were then placed on their ears, and no stimulation was given for 30 min. Intracolonic TNBS was applied to Group 4 (n = 9 Colitis-tVNS group) after anesthesia. Then, the clamps used in tVNS were placed on the ears, and stimulation was given for 30 min. TVNS application continued under anesthesia for ten days. At the end of day 10, the rats were sacrificed.

### 2.2 Animal Care Conditions

Rats were housed in a constant temperature (22 °C) and humidity-controlled environment on a 12:12 h light/dark cycle (lights turned on at 08:00 and turned off at 20:00) and fed with standard food and water ad libitum. A bark/sawdust cage was used to house the rats. Rats were allowed to acclimate to these conditions for at least 7 days before being included in the experiments.

### 2.3 Experiment Protocol

The colitis model is based on the classical trinitrobenzene sulfonic acid (TNBS) colitis model developed by Morris et al. (1989). TNBS was administered intracolonically into the distal colon through an 8-cm cannula from the anus. Saline was administered to the Sham group (Fig. 1). To ensure retention of TNBS in the colon, rats were kept in a head-down position for 2–3 min following intracolonic administration (Fig. 2).

### 2.4 Transauricular Vagal Nerve Stimulation (tVNS)

Transauricular VNS was applied by connecting electrodes to the tragus of both ears of anesthetized rats (ketamine 150 mg/kg and acepromazine 0.15 mg/kg, i.m.). Electrodes were also attached to the rats in the Sham group, but no stimulation was given. Transauricular VNS was performed for 30 min, twice daily (1 mA, 500 µs, 10 Hz, 5 V) for ten consecutive days using the Vagustim® tVNS device (Fig. 3).

### 2.5 Surgical Procedure

On the 10th day of the study, the rats were deeply anesthetized (ketamine 150 mg/kg and acepromazine 0.15 mg/kg,

**Table 1** Experimental groups

| | | | |
|---|---|---|---|
| GROUP 1 | 9 Sprague–Dawley albino male rats | Saline injection (day 1) | VNS – |
| GROUP 2 | 9 Sprague–Dawley albino male rats | Saline injection (day 1) | VNS + |
| GROUP 3 | 9 Sprague–Dawley albino male rats | TNBS injection (day 1) | VNS – |
| GROUP 4 | 9 Sprague–Dawley albino male rats | TNBS injection (day 1) | VNS + |



**Fig. 1** Intracolonic induction of TNBS in rats. (n = 36)



**Fig. 2** Rats were held in a head-down position following intracolonic TNBS administration. (n = 36)

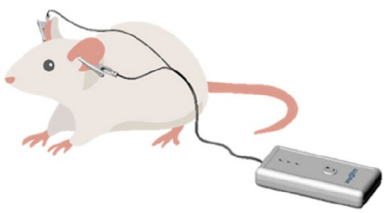


**Fig. 3** tVNS Device from Vagustim® company used in the experiment

i.m.). The rats were then administered saline transcardially. Colon tissues were removed.

### 2.6 Light Microscopic Method

Colon tissues of the experimental groups were fixed with 4% paraformaldehyde solution for light microscopic examination. After routine histological follow-up procedures, 4 µm thick sections were taken from paraffin blocks with a microtome (Leica RM 2245, Germany) for histochemical and immunohistochemical staining.

### 2.7 Hematoxylin&Eosin (H&E) Staining Protocol

Colon sections of the experimental groups were stained and closed by applying the Hematoxylin&Eosin (H&E) staining protocol. Images obtained from sections stained using a Leica DM 4000B (Germany) computer-aided light microscope were evaluated in the Leica LAS V 4.9 program.

### 2.8 Immunohistochemical Analysis

For immunohistochemical analysis, after deparaffinization and dehydration of colon tissues, antigen retrieval treatment was performed with citrate buffer (HDF, Turkey) (pH 6.0) at high temperature. The periphery of the washed tissues was limited with a tissue limiter pen (Pap pen). Tissues were washed 3 times for 3 min with PBS (Phosphate Buffer Saline) (Thermo, AP-9009–10, UK) (pH: 7.4). Then, 3% hydrogen peroxide (Thermo, TA-125-HP, UK) was applied for 10 min. Sections were washed with PBS and treated with UltraV block (Ultra Vision Detection System Large Volume Anti-Polyvalent, HRP, Thermo, TP-125-HP, UK) for 5 min. Sections were incubated overnight at + 4 °C with the appropriate dilution of Neuroligin-1 (Bioss, bs-4212R, 1:100) and Neurexin-2α (Bioss, bs-11104R, 1:100) primary antibodies. The sections were then washed 3 times with PBS for 3 min. After washing, a secondary antibody with biotin was applied for 10 min and washed again with PBS. After washing, the sections, which were left active for 10 min for the streptavidin peroxidase enzyme complex, were washed 3 times with PBS for 3 min, and an immune reaction was achieved by using chromogen containing chromogen diaminobenzidine (DAB) (Thermo, TA-125-HD, UK) substrate. Mayer's Hematoxylin (Thermo, TA-125-MH, UK) was used for floor paint. The washed sections were passed through increasing alcohol series and kept in xylol for 20 min. It was then closed with Entallan and viewed under a Leica DM 4000B (Germany) computer-aided light microscope using the Leica

LAS V4.9 (Germany) program. Twenty-30 ganglia per animal were photomicrographed at × 400 magnification, and percentages of immunopositive areas of Neuroligin-1 and Neurexin-2α primary antibodies in the myenteric plexus and number of positive cells were evaluated using the ImageJ (National Institutes of Health, USA) program [47].

### 2.9 Statistical Analysis

Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois) version 26.0 was used for data analysis. Since the study included less than 50 samples, the Shapiro–Wilk Test was used to examine the distribution of continuous variables. As a result of the test, it was determined that the data did not show normal distribution. The α level was defined as 0,05. Kruskal–Wallis Test and post-hoc Mann–Whitney U Test with Bonferroni Correction were used to examine the differences between groups. The significance level was determined as $p < 0,05$.

## 3 Results

### 3.1 Histopathological Analysis

Colon tissues of the Sham group and Sham + tVNS group exhibited normal microscopic appearance. Epithelium and crypt structures regularly appeared in the mucosa layer (Fig. 4). Tunica mucosa, submucosa, muscularis, and serosa layers were clearly distinguished. In the Colitis group, ulceration, intense inflammation dispersed through the mucosa, submucosa, and serosa layers, necrosis in some areas, and submucosal edema was noted. Epithelial and crypt integrity was observed to be lost. In the Colitis + tVNS group, histopathological changes were partially reduced, but lymphocyte infiltration and epithelial tissue loss were still present to some extent in some areas (Fig. 4).

In the colon histological images of the experimental groups, tissue integrity was maintained in Tunica mucosa **(Tm),** Tunica Submucosa **(Tsm),** Tunica muscularis **(Tmus),** Tunica serosa in Sham group and Sham + tVNS group, and epithelial and crypt integrity deterioration **(*),** inflammation **(▲),** ulceration **(►)** in Colitis group and Colitis + tVNS group. In the Colitis + tVNS group, a decrease in cell infiltration (*) and a decrease in microscopic tissue damage were observed compared to the Colitis group (n = 36) [Hematoxylin–Eosin (H&E), Scale bars: 200 µm. × 100, 100 µm. × 200].

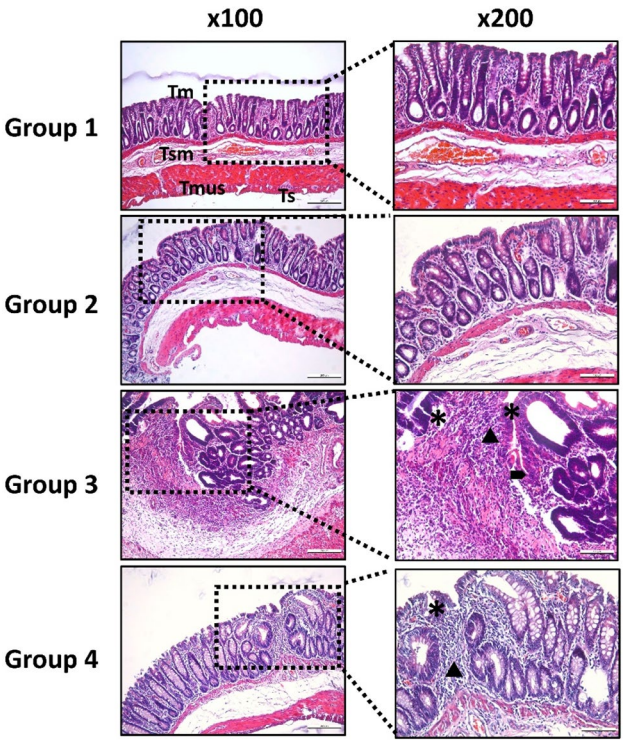



**Fig. 4** Colon histological images of the experimental groups. (n:36)

### 3.2 Immunohistochemical (IHC) Images of Experimental Groups

Examples of immunohistochemical images of positively stained Neuroligin-1 and Neurexin-2α cells in the myenteric plexus structures of 36 rats from 4 experimental groups are given in Figs. 5 and 6.

### 3.3 Between-Group Statistical Analyzes of Neuroligin-1 and Neurexin-2α Positive Cell Count

Neuroligin-1 and Neurexin-2α positive cell counts and immunoreactivity intensity percentages of all groups given in Table 2.

### 3.4 Neuroligin-1 Positive Cell Count

When the groups were compared in terms of the number of Neuroligin-1 positive cells, statistically significant differences were found between the Sham group and the Sham + tVNS group, the Sham + tVNS group and the Colitis group, the

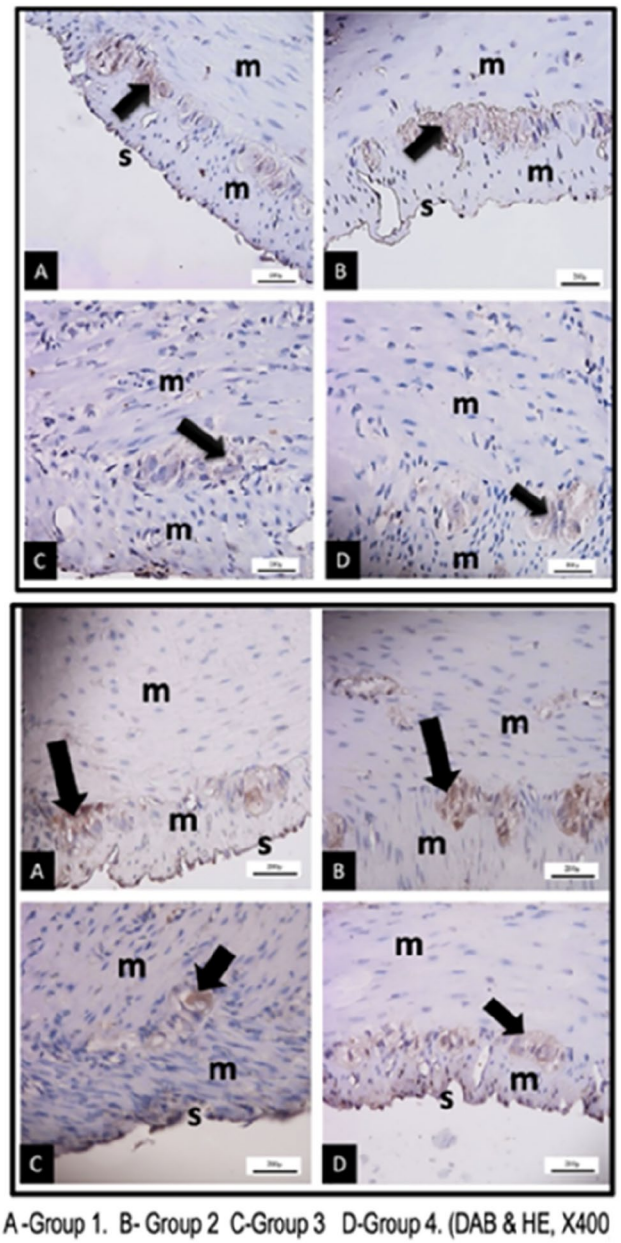



**Fig. 5** Neuroligin-1 immunohistochemical images of the groups, myenteric plexuses are marked with arrows, muscular layer **(m)** is shown with serosa layers **(s)** (n:36)

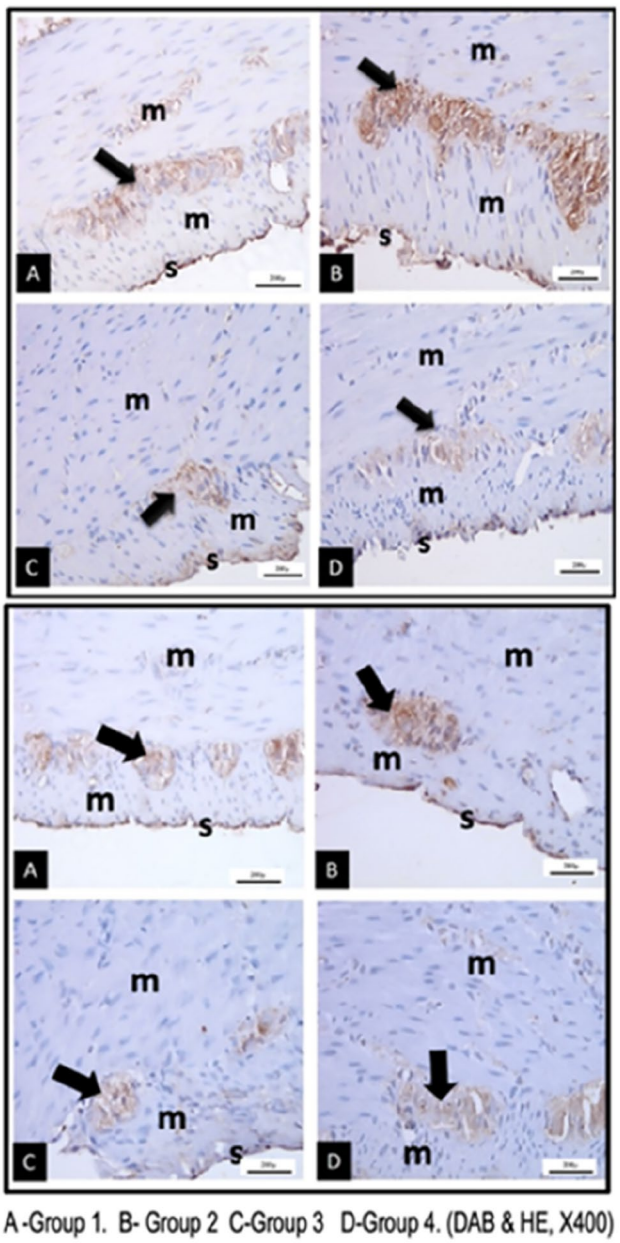



**Fig. 6** Neurexin-2α immunohistochemical images of the groups, myenteric plexuses are marked with arrows, muscular layer **(m)** is shown with serosa layers **(s)** (n:36)

Colitis group and the Colitis + tVNS group ($p < 0.05$). However, no statistically significant difference was found between the Sham group and the Colitis group, the Sham group and the Colitis + tVNS group, and the Sham + tVNS group and the Colitis + tVNS group (Fig. 5; Graph 1).

## 3.5 Neurexin-2α Positive Cell Count

When the groups were compared regarding Neurexin-2α positive cell counts, a statistically significant difference was found between the Sham + tVNS group and the Colitis group ($p < 0.05$). However, no statistically significant difference was found between the other groups (Fig. 6; Graph 2).

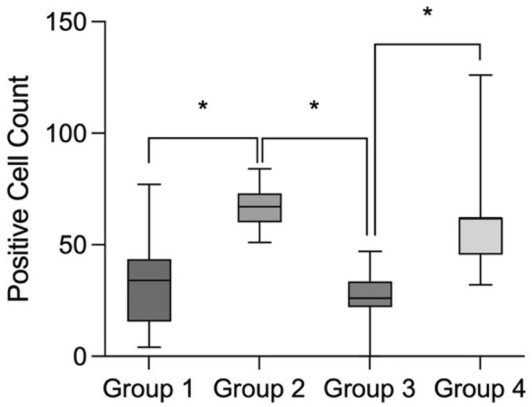
## 3.6 Intergroup Statistical Analysis of Neuroligin-1 and Neurexin-2α Immunoreactivity Intensity Percentages

### 3.6.1 Neuroligin-1 Immunoreactivity Intensity Percentages

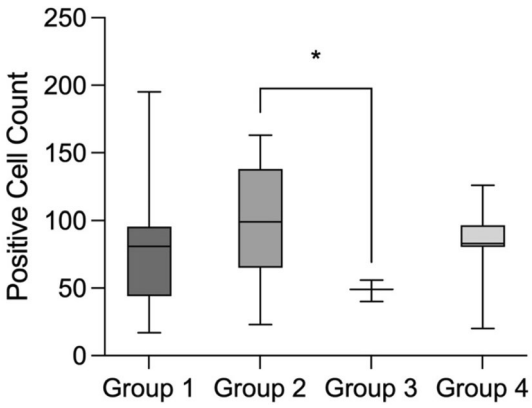
When the groups were compared in terms of Neuroligin-1 immunoreactivity density percentage, statistically

**Table 2** Neuroligin-1 and Neurexin-2α positive cell counts and immunoreactivity intensity percentages of all groups

| | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| Neuroligin-1 positive cell count | 34 (4–77) | 67 (51–84) | 26 (0–47) | 62 (32–126) |
| Neurexin-2α positive cell count | 81 (17–195) | 99 (23–163) | 49 (40–56) | 83 (20–126) |
| Neuroligin-1 immunoreactivity intensity percentages | 18.52 (10.37–37.36) | 17.260 (10.04–33.21) | 95.84 (7.20–23.55) | 12.44 (7.95–21.41) |
| Nurexin-2α immunoreactivity İntensity percentages | 35.90 (29.27–57.10) | 42.77 (30.58–57.40) | 33.10 (21.36–41.27) | 36.41 (17.60–47.27) |

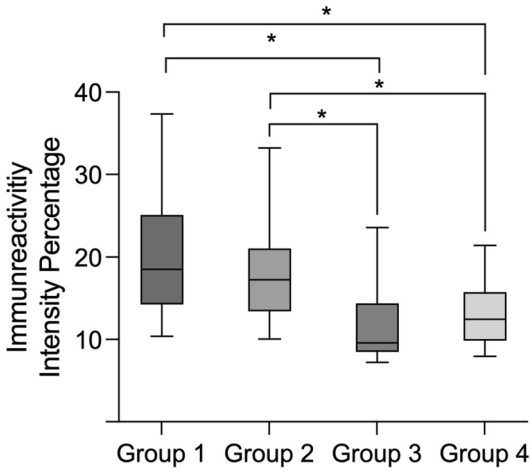



**Graph 1** Box graph showing Neuroligin-I positive cell counts in the experimental groups (*$p < 0.05$)




**Graph 2** Box graph showing Neurexin-2α positive cell counts in the experimental groups (*$p < 0.05$)

significant differences were found between the Sham group and the Colitis group, the Sham group and the Colitis + tVNS group, the Sham + tVNS group, and the Colitis group, and the Sham + tVNS group and the Colitis + tVNS group ($p < 0.05$). However, no statistically significant difference was found between the Sham group and the Sham + tVNS group (Fig. 5; Graph 3).



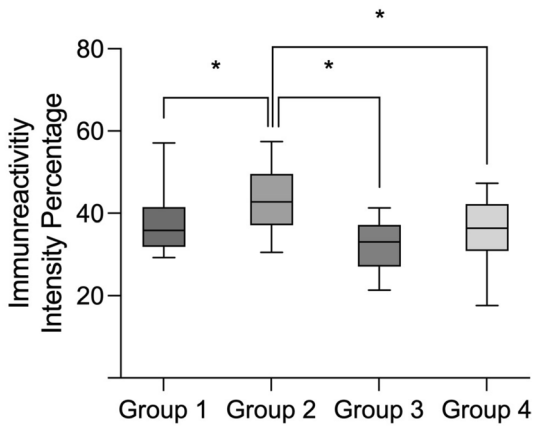

**Graph 3** Box graph showing the Neuroligin-1 immunoreactivity intensity percentages in the experimental groups. (*$p < 0.05$)

#### 3.6.2 Neurexin-2α Immunoreactivity Intensity Percentages

When the groups were compared statistically in terms of Neurexin-2α immunoreactivity density percentage, statistically significant differences were found between the Sham group and the Sham + tVNS group, the Sham + tVNS group and the Colitis group, the Sham + tVNS group and the Colitis + tVNS group ($p < 0.05$). No statistically significant difference was found between the other groups (Fig. 6; Graph 4).

## 4 Discussion

Inflammatory bowel disease is a common chronic disease with an unknown cause and no effective treatment. Studies have revealed changes in cell signaling molecules such as glial cell-derived neurotrophic factor, serotonin (5-hydroxytryptamine), endocannabinoids, and bacterial lipopolysaccharide during the formation of tissue inflammation in the colon. These signaling systems are thought to have neurogenic properties in the intestine [48–50]. It has been shown that the number of neurons decreases in colitis, neuronal death occurs, and with the resolution of inflammation, the




**Graph 4** Box graph showing the Neurexin-2α immunoreactivity concentration percentages in the experimental groups (*$p < 0.05$)

number of neurons partially recovers, and neurogenesis is also activated [51–54]. Targeting neurogenic pathways offers a promising path for developing new and innovative treatments for IBD [55].

Auricular transcutaneous vagus nerve stimulation (tVNS), a newly emerging neuromodulation therapy, has been proven to be safe and effective for many diseases such as epilepsy, major depressive disorders, insomnia, glucose metabolism disorders, pain, stroke, post-stroke rehabilitation, anxiety, fear, cognitive impairment, cardiovascular diseases, tinnitus, Prader-Willi syndrome, and COVID-19 [33]. Studies show that the therapeutic effect of tVNS is also promising in IBD [17–19]. There is relatively little literature on its mode of action and functionality. Studies are conducted to investigate the therapeutic effect of tVNS in IBD, but its mechanism of action has not yet been fully explained. Therefore, more studies are needed. In line with this need, we examined the anti-inflammatory effects of tVNS and its impact on ENS synapse formation in the experimental colitis table we created with TNBS. As a result of the study, we found an improvement in inflammation with tVNS in the colon tissue in the colitis group, a decrease in ENS synaptic protein amounts in the colitis group, and an increase in the groups that received tVNS. We have not encountered a similar study on ENS synaptic proteins in colon tissue that has been conducted before. The limitations imposed on the number of animals used in experimental studies due to ethical principles are one of the limitations of our study. Data obtained as a result of studies using larger samples will support both our study and the literature. The limited number of studies in the literature regarding the optimal features, amplitudes and frequencies of the device and electrodes used for tVNS application, as well as the paucity of literature information regarding the determination of the optimal stimulation duration and appropriate stimulation areas required for the application are other limitations that challenge us. Although these limitations make it difficult to determine the parameters we will apply during the design of our study, we also believe that our study will contribute to the knowledge of the literature on these issues.

In a study conducted by Meregnani et al., low-frequency (5 Hz) left cervical VNS was applied to rats with TNBS-induced colitis for 5 days, 3 h per day. Inflammation in the colon tissue was improved in rats receiving left cervical VNS [56]. Lei Tu and colleagues applied Sacral Nerve Stimulation (SNS) to rats with TNBS-induced colitis for 10 days, starting five days after TNBS induction. SNS reduced disease activity index, microscopic and macroscopic scores, myeloperoxidase activity, and proinflammatory cytokines (TNF-α, IL-6). In addition, SNS was observed to increase acetylcholine and parasympathetic activity in colon tissues and to regulate the activity of the ENS and immune system [57]. Pasricha and colleagues induced colitis with oral Dextran Sodium Sulfate (DSS) and applied SNS. In conclusion, SNS has been shown to promote clinical recovery within 5 days after treatment, improve myeloperoxidase activity, histological changes in tissue, TNF-α levels, and increase M2 macrophage population. SNS has been shown to increase vagal activity and decrease sympathetic activity significantly. It has also been shown to modulate neurotransmitters involved in systemic inflammation and regulate sympathovagal balance [58].

Ghia and colleagues conducted a study to determine whether the vagus nerve is anti-inflammatory in a DSS-induced colitis model. Vagotomy was performed in mice with colitis. Inflammation was observed to be exacerbated and accelerated in the vasectomized mice. The vagus nerve has been shown to reduce chronic colitis through a mechanism derived from macrophage colony-stimulating factor. The vagus nerve has also been shown to protect against acute relapse in chronic inflammation [59].

Jin and colleagues established a colitis model in TNBS rats. Electroacupuncture was applied to rats with electrodes placed at the ST36 acupuncture point, and its effects were examined. After electroacupuncture, a significant decrease was observed in mucosal damage such as edema, hyperemia, intestinal wall thickening, mucosal erosion, and ulcers in the colon. When the electroacupuncture and control groups were compared, a significant decrease in histological scores was observed. It was determined that electroacupuncture (at ST36) improved colon inflammation by inhibiting proinflammatory cytokines through an autonomous mechanism [18].

Our study's histological evaluation results showed that the colon tissues of the Sham and Sham + tVNS groups had standard histological structure. In the colon tissue of the colitis group, ulceration, intense inflammation including the mucosa, submucosa, and serosa layers, necrotic changes in some areas, and submucosal edema were seen. It was observed that the integrity of the epithelium and crypts was

lost. When we look at the Colit + tVNS group, it was seen that histological changes decreased, lymphocyte infiltration, and epithelial tissue loss partially continued in some areas. In line with these results, we obtained results that support the above-mentioned studies and that tVNS reduces inflammation with its anti-inflammatory effect in colitis.

In another study we conducted previously, tVNS showed a significant improvement in the inflammatory response in colon and brain tissue, a decrease in proinflammatory cytokines (IL-1β, IL-6, and TNF-α'), and an increase in the levels of the anti-inflammatory cytokine IL-10 in TNBS-induced colitis. TNBS-mediated colon inflammation and tissue damage were shown to be associated with neuroinflammatory responses in brain tissue. In addition, it was shown for the first time that tVNS suppressed the neuroinflammatory response in the gut-brain axis in an IBD model [60].

The most widely studied trans-synaptic junction complexes that direct synaptogenesis and support the maintenance and stability of the mature terminal are assemblies of presynaptic Neurexins and postsynaptic Neuroligins. Fluctuations in the levels of Neuroligins and Neurexins can affect the balance between excitatory and inhibitory neurotransmission in the brain and damage synapses and dendrites [39]. Neuroligins and Neurexins coordinate synaptogenesis by secreting proteins for signal transmission between neurons [37, 61]. The transsynaptic Neurexin-Neuroligin complex participates in the formation of functional synapses in vitro. Many studies have been conducted on the functional organization of synapses via $Ca^{2+}$ channels in the CNS [62, 63]. Some studies have found that diseases with cognitive disorders, such as autism spectrum disorder and schizophrenia, are associated with the breakdown of Neurexin and Neuroligin [38, 64]. Mutations in genes encoding synaptic proteins such as Neuroligin 3 have been associated with autism. Gastrointestinal dysfunction is often reported by individuals diagnosed with autism spectrum disorder, but the cause remains unknown [65]. Synapses and neuronal circuits are a vital building block of the functional ENS. The interaction of Neurexin and Neuroligin induces the differentiation of both presynaptic and postsynaptic terminals and enables synaptogenesis of excitatory and inhibitory synapses, thus maintaining homeostasis in vitro [33].

In the study conducted by Wang et al., it was shown that the expression levels of Neuroligin 1 and Neuroligin 2 gradually increase with embryonic growth and that there is a temporal correlation with ENS development at a later embryonic stage [42].

Srdan Sanovic and colleagues induced colitis in rats with dinitrobenzene sulfonic acid (DNBS) and examined the number and distribution of enteric neurons. Bloody diarrhea, severe mucosal damage, submucosal ulceration, and significant inflammatory cell infiltrates were observed on day 6. While the number of ganglia in the myenteric plexus did not change, a substantial decrease in the number of neurons was found in the inflamed area of the colon [45]. It has been suggested that neuronal loss within the ENS is a fundamental consequence of intestinal inflammation and may be a major cause of the post-enteritis dysfunction seen in IBD in humans [66].

Hirschsprung disease is ENS's most characteristic congenital anomaly, characterized by the absence of all ganglia of variable length in the terminal part of the intestine and mutations in multiple genes [67]. Study by Leembruggen et al. found that the levels of synaptic proteins Neuroligin and Neurexin in the ENS were significantly downregulated in the aganglionic and transitional segments of Hirschsprung patients compared to regular segments [65].

In a study by Filova and colleagues, the effects of oxytocin treatment applied during the neonatal period on synaptic proteins in the hippocampus were examined. Oxytocin treatment was applied to rats on P2-P3 days after birth. As a result, a decrease in the density of Neuroligin 3 positive cells in the Ca3 field in the hippocampus was detected. No effect was observed in Neurexin 2α gene expression, and a decrease in Neurexin 2β levels was observed. No change was observed in Neuroligin 1 and Neuroligin 2 levels. Increases in oxytocin, Neuron Specific Enolase mRNA levels, and decreases in glial fibrillary acid protein mRNA levels, which is a marker of astrocytes, were detected. These results suggested that synaptic proteins may be controlled by oxytocin in the early stages of brain development. This study shows that changes in cell adhesion molecules and synapse protein levels may be related to the modulation of neuronal cell number [68]

As a result of our study found that the number of Neuroligin-1 and Neurexin-2α positive cells decreased in colitis groups compared to sham groups and increased in both tVNS sham and colitis groups. We had results that did not show a statistically significant difference in the number of Neurexin-2α positive cells. We thought this could be due to the lack of sufficient sample size. We observed that the percentage of Neuroligin-1 and Neurexin-2α immunoreactivity decreased in colitis groups compared to sham groups and increased in both tVNS sham and colitis groups. There was no statistically significant difference between the colitis groups and the colitis + tVNS groups. One result we could not understand was a substantial decrease in the percentage of Neuroligin-1 immunoreactivity in the sham + tVNS group. These results suggest that tVNS supports neuronal recovery while treating inflammation in the colon. Therapies that promote enteric neurogenesis may offer clinicians new and powerful therapeutic alternatives to treat gastrointestinal injury.

## 5 Conclusion

Our study is the first study in the literature to show the effects of tVNS on the ENS in terms of the stimulation parameters we used. The significant IHC results we obtained as a result of the study suggest that tVNS improves inflammation in the colon tissue and activates enteric synaptogenesis by increasing synaptic proteins. However, more rigorous and systematic studies are needed to investigate the impact of stimulation parameters, stimulation sites, and electrode types on brain activation and clinical outcomes. Using neuromodulation with bioelectronic devices as a therapy is an emerging field in bioelectronic medicine. tVNS can be used as an alternative non-pharmacological treatment to traditional treatment methods or in combination with conventional treatments. Treatments that support enteric neurogenesis may provide clinicians with new and powerful therapeutic options for the treatment of IBD.

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## Declarations

**Conflict of interest** All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript. The authors declare no competing interests.

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