

The Effects of Ionotropic GABA Receptor Blockage on Brain in Sepsis-induced Rats

Gulten Ates (✉ gultenates89@gmail.com)

Istanbul Yeni Yuzyil University <https://orcid.org/0000-0001-8675-9031>

Elif Ozkok

Istanbul University: Istanbul Universitesi

Gul Ipek Gundogan

Yeni Yuzyil Universitesi

Sule Tamer

Istanbul University, Istanbul Medical Faculty

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Abstract

Encephalopathy develops following sepsis, which is defined as life-threatening organ failure due to the irregular response of a host to infection. It has high mortality and morbidity rates. In this study, we aimed to investigate the effects of inflammation on brain tissue, and the effects of the GABA_A receptor antagonist bicuculline in rats with sepsis. Sepsis was experimentally generated in rats using Lipopolysaccharide (LPS). The rats were divided into four groups as control, LPS (10 mg/kg i.p.), bicuculline (1.5 mg/kg bicuculline methiodide s.c.), and LPS+bicuculline. Electrophysiologic recordings and body temperature measurements were completed at the 24th hour after injection, and blood samples were taken from the heart for measurements of biochemical parameters. TNF- α , IL-10, and GABA levels were measured using ELISA, and MDA levels were measured using the Bouge method from tissue. Tissue imaging was performed with S100- β , NEUN, and synaptophysin antibody using immunofluorescence staining. One-way ANOVA and the Tukey test were used in statistical analysis. Inflammatory parameters significantly increased in brain tissue in the LPS group compared with the other groups. The immunofluorescence staining results in brain tissue were as follows: S-100 β involvement increased, and NeuN and synaptophysin involvement decreased in the LPS group. In electrophysiologic recordings, activity consistent with acute non-focal seizures was observed in the LPS group; however, resting status in others. We suggest that the GABA_A antagonist bicuculline methiodide may be a prophylactic agent in sepsis, which caused the impaired neurotransmitter balance, increased pro-inflammatory cytokine and lipid peroxidation, and decreased anti-inflammatory cytokine levels.

Introduction

Sepsis and its complications are among the main causes of mortality in approximately 50% of deaths in intensive care units (ICUs) (Fleischmann-Struzek et al., 2020; Moraes et al. 2021). In recent years, inflammatory cascades that cause death with multiple organ failure have been extensively investigated. A large number of studies focused on peripheral organs such as the lung, liver, intestine, and kidney; however, few studies investigated brain during sepsis. Septic encephalopathy represents central nervous system (CNS) dysfunction due to sepsis and systemic inflammatory response syndrome (SIRS) (Kumar 2020, Dal-Pizzol et al. 2014). In addition, encephalopathy, which is the most common finding in ICUs, has a high mortality rate in patients with sepsis (Dal-Pizzol et al. 2014, Husabo et al., 2020). Studies on pathophysiologic mechanisms that lead to septic encephalopathy are ongoing.

Microorganisms or various toxin-induced macrophage release pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), and interleukins (ILs), which induce an endotoxic response. The environmentally produced inflammatory signal stimulates endogenous expression of pro-inflammatory cytokines of brain cells by crossing the blood–brain barrier (Weil et al. 2010). During septic encephalopathy, proinflammatory cytokines have been shown to increase the activity of gamma (γ)-amino butyric acid (GABA) in the CNS (Rossi et al. 2011). The ionotropic and metabotropic receptors of GABA are commonly found in the CNS, including those receiving cardiovascular and autonomic control. In hepatic encephalopathy and endotoxic shock, inflammatory molecules such as nuclear factor- $\kappa\beta$ and

mitogen-activated protein kinase ERK (MAPKerk), showed an increase in GABA receptor activity and in the upregulation of central GABAergic transmission in cardiovascular and inflammatory reactions (Hellstrom et al. 2005; Serantes et al. 2006). In studies, the stimulation of macrophage GABA receptors in experimental animals with endotoxemia was reported to activate nuclear factor- κ B and MAPKerk pathways. Bicuculline is a well-known blocker of the GABA_A receptor, and studies showed that lipopolysaccharide (LPS) was reduced with both hypotensive and inflammatory effects in studies where the receptor was blocked by bicuculline. In addition, bicuculline was reported to inhibit acetylcholinesterase in the literature GABA_A receptor antagonists were reported to be effective on septic brain tissue (Sallam et al. 2006). However, few studies have tried to describe the molecular mechanism.

In the present study, we aimed to investigate the effects of ionotropic GABA_A receptor blockage in brain tissue on molecular, morphologic, and electrophysiologic levels in rats with LPS-induced sepsis.

Materials And Methods

Experimental Groups:

Ethics committee approval for the study was obtained from the Local Ethics Committee of Animal Experiments of Istanbul Bagcilar Training and Research Hospital (2017/63).

Male adult *Sprague dawley* rats weighing 180-230 g were divided into four groups as controls (n=8), LPS (n=8) (10 mg/kg, i.p.), bicuculline (n=8) (1.5 mg/kg, s.c.), and LPS+bicuculline (n=8). The animals were fed with a commercial diet and tap water ad libitum, housed in cages kept at a controlled temperature (22±2°C) and humidity (55-60%) with a 12-hour light/dark cycle.

Experimental Procedures:

LPS from *Escherichia coli* O55: B5(Sigma Aldrich, Product No: L2880) was dissolved in 1 mL of sterile saline solution, and a single intraperitoneal dose of 10 mg/kg was injected. Bicuculline methiodide was dissolved in 1 mL of sterile saline solution, and a single subcutaneous dose of 1.5 mg/kg was administered. Animals were anesthetized using ketamine (90 mg/kg) (i.p.) + xylazine (10 mg/kg) (i.p.) 24 hours after the LPS injection. Under anesthesia, non-invasive electrophysiologic recordings were performed, and rectal body temperatures were measured. Blood samples were taken from the heart, and the animals were sacrificed. To investigate the levels of TNF- α , IL-10, and GABA using enzyme-linked immunosorbent assay (ELISA), and also to determine malondialdehyde (MDA) levels using the Bouge method total brain tissues homogenized and also for each group, total brain tissue placed in 10% formaldehyde for histopathologic evaluations.

Measured of GABA, TNF- α , IL-10 Level:

The total GABA (YH-Biosearch, Shanghai, China), TNF- α (YH-Biosearch, Shanghai, China), and IL-10 (YH-Biosearch, Shanghai, China) levels were measured in homogenized brain tissue using specific

ELISA method using with biotin-based double antibody sandwich technique assay at 450 nm.

Lipid peroxidation procedure:

Lipid peroxide levels in homogenized brain tissue were determined by measuring the MDA levels using the Bouge method. In this method, the end products of lipid peroxidation were spectrophotometrically measured using thiobarbituric acid (TBA, Sigma, Aldrich) in warm, acidic conditions, read at 540 nm.

Immunofluorescence protocol:

Brain tissue was fixed in 10% formaldehyde overnight. In follow-up procedures, tissue was processed to remove any remaining fixative in a series of decreased alcohol concentration solutions for 24 hours. The alcohol was removed from the tissue samples, which were then treated with xylene for 1 hour to ensure good penetration of paraffin wax. Tissues were kept in 58°C pure paraffin for 2 hours. When the tissues were ready for blocking, they were embedded in paraffin. For staining, 10-μm-thick sections were taken from the blocks using a microtome and transferred to poly-L-lysine-coated slides. To detect degeneration in neurons, S100B antibody (orb388636) biorbyt, Unipoert ID: P04271, Entrez: 6285, Mouse, (secondary antibody Dylight 649, goat anti-mouse IgG (H&L) Abbkine # A23610) was used. To detect living neurons, NeuN antibody (orb48522) biorbyte, Unipoert ID: A6NFN3, Entrez: 146713, Rabbit (secondary antibody green-FITC) was used. To detect synaptophysin, SYP (orb69251) biorbyte, Entrez: 6855, Mouse (secondary antibody Dylight 649, goat anti-mouse IgG (H&L) Abbkine # A23610) was used. Multiple fluorescence staining was performed on the sections. After the sections were purified with paraffin, xylene was evaporated until the sections became white. Subsequently, the sections were allowed to stand for 10 minutes in cold methanol at -20°C. Finally, for rehydration, the sections were immersed in distilled water for 2 minutes. The epitopes were then incubated with 0.2% Tween 20 for 5 minutes and washed with distilled water, and then boiled in microwave oven with 1 x citrate buffer (citrate buffer, pH 6.0) for 20 minutes. Sections were washed twice at room temperature for 10 min using phosphate-buffered saline (PBS) (pH 7.6). Prior to the primary antibodies being optimized for dilution, NEUN (diluted with PBS containing 2% serum) was dropped onto the sections, which were then covered with a coverslip and kept overnight at + 4°C. The next day, the NEUN secondary antibody was incubated at room temperature for 2 hours, washed with PBS, and then dropped onto sections with SYN (diluted with PBS containing 10% goat serum) or S100 (diluted with 0.3% TritonX-100, PBS containing 0.3% serum), covered, and kept at + 4°C for one night. The following day, the secondary antibodies of the primary antibodies were incubated at room temperature for 2 hours and washed with PBS. The core dye was incubated with Dapi and the preparations were sealed using anti-fading and mounting medium. Histologic changes were evaluated and photographed using fluorescence microscopy with FITC, H&L, and DAPI-compatible filters.

Statistics:

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 21.0 Statistical Software (SPSS, Inc., Chicago, IL, USA). All parametric data were evaluated by Oneway analysis of variance (ANOVA) test. The post-hoc Tukey test was used to determine the group that caused the difference. Data were presented as the mean \pm Standard error of the mean (SEM). The level of significance was accepted as $p<0.05$.

Results

Findings of septic conditions:

In the LPS group, the animals were detected to be either hypothermic ($34.7^{\circ}\text{C} \pm 0.4$) or hyperthermic ($38.8^{\circ}\text{C} \pm 0.7$) in accordance with their metabolic tendencies. The body temperatures of animals in the bicuculline, control, and LPS+bicuculline groups were determined in standard values.

In the LPS group, the leucocyte count was found significantly higher compared with the bicuculline ($P<0.01$), control and LPS+bicuculline ($P<0.05$) groups. There were no significant differences between the other experimental groups ($P>0.05$) (Fig.1).

Cytokine findings:

In the LPS group, the TNF- α level was significantly higher than control and bicuculline groups ($P<0.05$). There were no significant differences between the other experimental groups ($P>0.05$) (Fig.2-A).

IL-10 levels were significantly decreased in the LPS group compared with the control ($P<0.05$). There were no significant differences between the other experimental groups ($P>0.05$) (Fig.2-B).

GABA ELISA findings:

In the LPS group, GABA levels were found higher compared with levels in the other groups. However, the result was not significant ($P>0.05$) (Fig.2-C).

Lipid peroxidation findings:

MDA levels were significantly increased in the LPS group compared with the control and LPS+bicuculline groups ($P<0.05$). There were no significant differences between the other experimental groups ($P>0.05$) (Fig.2-D).

Immunofluorescence findings:

In the LPS group, S100- β immunoreactivity which were marked degenerated neurons was found higher; however, NeuN which were marked living neurons and synaptophysin immunoreactivity were found lower than in the other experimental groups ($P<0.01$). No significant changes were detected between the other groups ($P>0.05$) (Fig.4-9).

Neuron-specific enolase (NSE) findings:

In the LPS group, NSE levels were significantly higher than the levels in other groups ($P<0.01$). No significant change was detected between the other groups ($P>0.05$) (Table.1).

Electrophysiologic findings:

In the LPS group, significant increases were detected in the amplitudes of the electroencephalography waves at the min-max value ($P<0.001$). No significant changes were detected between the other groups ($P>0.05$). In recordings, activity consistent with acute non-focal seizures was observed in the LPS group; however, resting status in others (Table.2).

Discussion

In our study, we aimed to investigate the effects of inflammation on the brain tissue of rats with LPS-induced sepsis, and how these effects could be changed using an ionotropic GABA_A receptor antagonist, also acetylcholinesterase inhibitor-bicuculline. The mechanism of neurologic complications in sepsis-induced encephalopathy has not been fully elucidated. Researchers in many studies reported that the brain GABA receptor density and serum GABA levels were increased in experimental septic encephalopathy models, bacteria were reported to possibly produce large amounts of GABA, and bacterial sepsis was a cause of encephalopathy. Furthermore, recent studies reported that a GABA agonist, benzodiazepine exacerbated septic encephalopathy (Jones et al. 1984; Minuk et al. 1985; Sallam et al. 2006; Ramirez- Jarquin et al. 2017; Shulyatnikova et al., 2020).

Acute brain dysfunction was reported in LPS-induced sepsis, especially with the increase in GABA levels in the hippocampus, and with a decrease in the level of consciousness and neuronal activity (Kadoi et al. 1996; Hellstrom et al. 2005; Serantes et al. 2006; Jacob et al. 2011; Malaguarnera et al. 2019). Rossi et al. reported that the released proinflammatory molecules increased the central GABA activity during autoimmune encephalomyelitis (Rossi et al. 2011). Sugiura et al. showed that macrophage GABA receptors increased the susceptibility of mice to endotoxemia through regulation by increasing the NF κB, and MAPK/ERK (Sugiura et al. 2011).

For blocking GABAergic activity using the GABA_A receptor blocker bicuculline, and GABA_B receptor blocker baclofen, Salam et al. showed that administration of bicuculline improved cardiac, hemodynamic, and inflammatory findings compared with a baclofen-treated group, and ionotropic GABA_A receptors were reported to be more effective in the inflammatory process (Sallam et al. 2006).

Variable levels of neurotransmitter in sepsis led to alterations of cognitive processes, in addition to the damage of neuronal mechanisms and brain tissue (Ramirez- Jarquin et al. 2017; Malaguarnera et al. 2019). Inflammatory mediators were reported to cause increased receptor activity by binding to GABA_A receptors by modulating the expression of GABA_A receptor subunits with infectious factor, hypoxia or stress (Wang et al. 2012). In our study, we observed that GABA levels increased in total brain tissue;

however, the increase was not statistically significant ($p>0.05$). In the bicuculline and LPS+bicuculline group, we found similar GABA levels to those in the control group.

In their experimental study, Winder et al. observed that GABA levels were elevated in the brain tissue of rats with sepsis. They reported that GABA levels increased, especially in the sub-brain region; however, in a total brain investigation, this increase was not as significant as in our study (Winder et al. 1988). Our findings of GABA levels in total brain tissue were similar with the levels in other studies in the literature.

Recent studies showed that acetylcholine and bicuculline methiodide could activate nicotinic acetylcholine receptors (Hsu et al. 2004; Thomsen et al. 2012; Ramírez-Jarquín et al. 2017). Wang et al. demonstrated that activation of nicotinic acetylcholine receptors inhibited TNF- α synthesis and decreased inflammatory response by increasing IL-10 release (Wang et al. 2003). In addition, activation of nicotinic acetylcholine receptors was reported to be associated with a decrease in the production of NO and reactive oxygen species (Li et al. 2000; Hoover et al. 2017). Bicuculline methiodide was suggested to have a protective effect after cecal ligation and perforation in a study examining the change of cytokines with bicuculline methiodide. TNF- α is known to trigger the production of free radicals in neonatal rat hepatocytes and reduce oxidative metabolism (New et al. 2001). This activity is positively controlled by various up-regulatory stimuli, including TNF- α and IL-1 β , and is negatively controlled by pro-inflammatory cytokines such as IL-10 (Szabo et al. 1993; Thiemann et al. 1993).

In our study, we investigated the effects of bicuculline methiodide on IL-10 levels from TNF- α and anti-inflammatory cytokines from proinflammatory cytokines in brain tissue in the sepsis group. The TNF- α levels of the bicuculline and LPS+bicuculline groups were in close proximity to the control group. We observed that TNF- α levels increased in total brain tissue in the sepsis group ($P<0.05$). TNF- α is known to reach peak values at minute 90 and its half-life is shorter. In addition, studies showed that TNF- α concentrations were decreased by stimulating a number of pathways such as anti-inflammatory pathways in long-term sepsis models (Thiemermann et al. 1993). We found that IL-10 levels significantly decreased in the sepsis group compared with the other experimental groups ($P<0.05$). Our other experimental groups were similar to the control group. The decrease in IL-10 levels was reported as an indicator of the inflammatory pathway induced by TNF- α in the literature (Munford et al. 2005). Although the NF- $\kappa\beta$ pathway plays an important role in the regulation of cytokine production, it has been supported in studies that bicuculline methiodide might decrease proinflammatory cytokine production by inducing activation of nicotinic acetylcholine receptors instead of using the NF- $\kappa\beta$ pathway (Loop et al. 2003). However, researchers reported that nicotinic acetylcholine receptors might be associated with the anti-inflammatory effect of bicuculline methiodide in sepsis (Wang et al. 2003). In another study, bicuculline methiodide was suggested to inhibit the pro-inflammatory cytokine release through nicotinic α -7 receptors (Sallam et al. 2006; Wang et al. 2003). We observed that TNF and IL-10 cytokine values decreased in the sepsis group in the LPS+bicuculline group. This may be explained by the fact that bicuculline blocks GABA_A ionotropic receptors and inhibits acetylcholinesterase and blocks the inflammatory process. The alpha-7 nicotinic Ach receptor agonist GTS-21 was reported to decrease cytokine levels in studies

conducted with volunteers who were given endotoxin treatment. Some studies reported that cholinergic neurons were particularly sensitive to systemic inflammation (Dal-Pizzol et al. 2014). These findings are in line with our results. Bicuculline methiodide, which is known to have acetylcholinesterase inhibition, was suggested to possibly be a good anti-inflammatory agent as a result of stimulating cholinergic pathways, and was supported by other studies (Sallam et al. 2006; Hsu et al. 2004; Thomsen et al. 2012; Malaguarnera et al. 2019). Increased ROS production in sepsis causes neuropathy and myopathy leading to destruction of brain and tissue. In studies with lipid peroxidation in sepsis, thiobarbituric acid reactive products were analyzed, and increased MDA levels were reported to have effects such as impaired blood-brain barrier (Martins et al. 2003). In our study, we observed that MDA levels in brain tissue increased in the sepsis group compared with the control and LPS+bicuculline groups ($P<0.05$). In experimental studies, MDA levels were reported to increase in the cortex, hippocampus, and cerebellum at hour 6 in sepsis-induced rats (Takezawa et al. 1983). A significant increase was shown in MDA levels in the sepsis group.

High serum levels were reported in cases of neuron-specific enolase (NSE) levels in the brain quantification of cardiac injury, cardiac arrest in the clinic, septic shock, brainstem, neuroendocrine tumors, and malignancy (Isgro et al. 2015). Researchers reported that NSE levels in patients with sepsis in ICUs could be used to predict the clinical course and might show high sensitivity and specificity (Weigand et al. 2000). Yao et al. reported that NSE and S-100 β levels were significantly higher in patients with septic encephalopathy (Isgro et al. 2015; Yao et al. 2014). In our study, we found that NSE levels significantly increased in the sepsis group compared with the other experimental groups ($P<0.01$). Our findings support the data in the literature.

We obtained non-focal epileptic wave-like recordings from our rats with sepsis under mild anesthesia in an investigation of the non-invasive total brain electrical activity records. We found a higher degree of significance in the LPS group compared with the other groups in investigations of the minimum-maximum ratios of wave amplitudes between the groups. The other experimental groups had similar wave characteristics and minimum-maximum ratios to the control group. In our group, where we used bicuculline methiodide, we detected no convulsion waves, supporting that it may be a suitable agent in GABA blockade according to other GABA antagonists and bicuculline derivatives, in accordance with the literature (Sallam et al. 2006).

In the immunofluorescence staining of the neurotransmitter through the synaptophysin antibody, which is a specific glycoprotein for the synaptic terminals, we observed a small involvement in the brain tissue in the sepsis group compared with the other groups. This shows that neurotransmitter regulation was impaired in the sepsis group. These results indicate that the rats are also affected by sepsis. We observed that the results were similar in the bicuculline and LPS+bicuculline groups. This suggests that bicuculline had a positive effect on the neurotransmitter regulation of sepsis.

S100- β is one of the markers used to demonstrate neuronal damage in the brain. Studies have shown increased serum levels of S100- β in sepsis, brain trauma, cerebral stroke, hypoxic ischemia, and

encephalopathy. The exact mechanism for secretion of S100- β has not been fully elucidated; however, it was reported to cause neural tissue damage and was associated with oxidative stress (Isgro et al. 2015; Yao et al. 2014).

In the comparison of the number of damaged cells stained with S100 β antibody in the sepsis group and the number of cells stained with NeuN antibody, which stains live neurons, we observed increased counts of live neurons in our study in the control, bicuculline, and LPS+bicuculline groups compared LPS group. Our findings suggest that LPS causes neuronal damage, and bicuculline reduces this damage. However, detailed studies are required to explain the mechanism (Kruiff et al. 2011; Hamed et al. 2009).

Conclusion

In conclusion, we suggest that the GABA_A antagonist bicuculline methiodide, which is known as an acetylcholinesterase inhibitor, can be used a curative agent on inflammatory processes observed in d sepsis induce encephalopathy such as imbalanced neurotransmitter levels, increased pro-inflammatory cytokine levels, decreased anti-inflammatory cytokine levels, and increased lipid peroxidation levels in brain tissue.

Declarations

ETHICAL STATEMENT:

Ethics approval and consent to participate:

Ethics committee approval for the study was obtained from the Local Ethics Committee of Animal Experiments

of Istanbul Bagcilar Training and Research Hospital (2017/63).

Consent to Participate

Not applicable to this article, since data of this study do not involve human participants.

Consent for Publication

Not applicable to this article, since data of this study do not involve human participants.

Authors' contributions:

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. GA, EO and ST conceived and designed the experiments; GA, EO, GIG and ST performed the

experiments; GA wrote the original manuscript; ST and EO reviewed and edited the manuscript.

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Competing interests:

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data Availability Statement:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table.1: NSE levels between the all of experimental groups

	Control	LPS	Bicuculline	LPS+Bic
(μ g/L)	0,23±0,398	3,5675±1,325 ^{a,b}	0±0	0,206±0,221

Table.2: EEG waves max.min levels between the all experimental groups

Groups	Max.-Min.	±Sd
Control	1.1433	0,0513
LPS	10.2397**	0,9853
Bicuculline	0.8334	0,1266
LPS+Bic	1.255	0,203

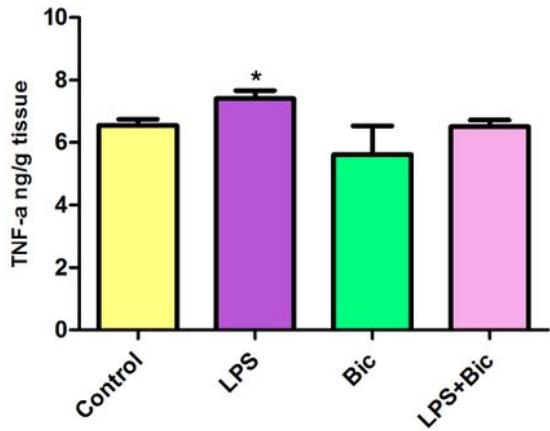
Figures

Figure 1

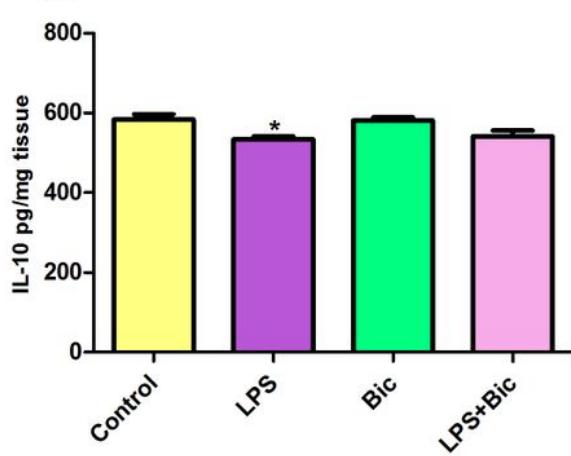
Leukocyte counts of experimental groups

*: p<0,5 **: p<0,01

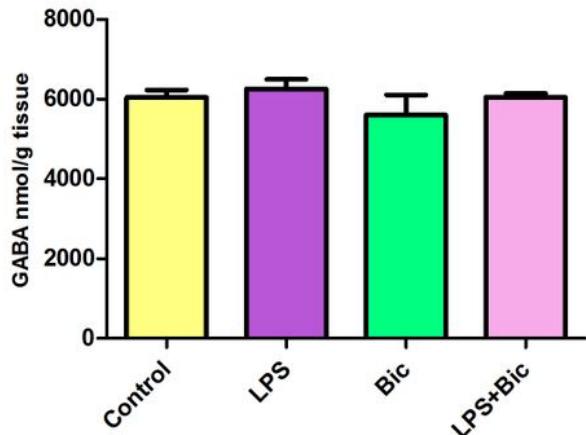
A



B



C



D

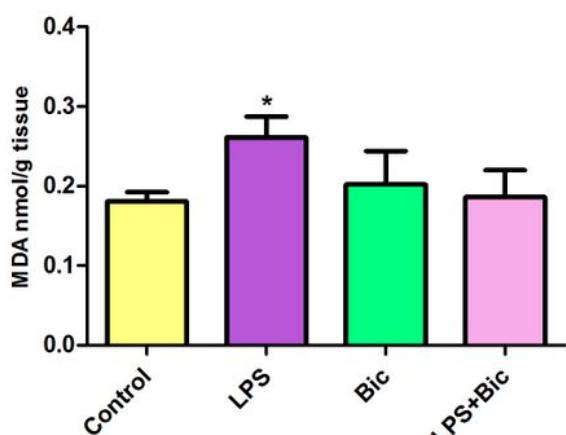


Figure 2

A-B: TNF- α ve IL-10 levels of experimental groups in brain tissue

*: p<0,5 LPS vs. other experimental groups

C-D: GABA ve MDA levels of experimental groups in brain tissue

*: p<0,5 LPS vs. other experimental groups

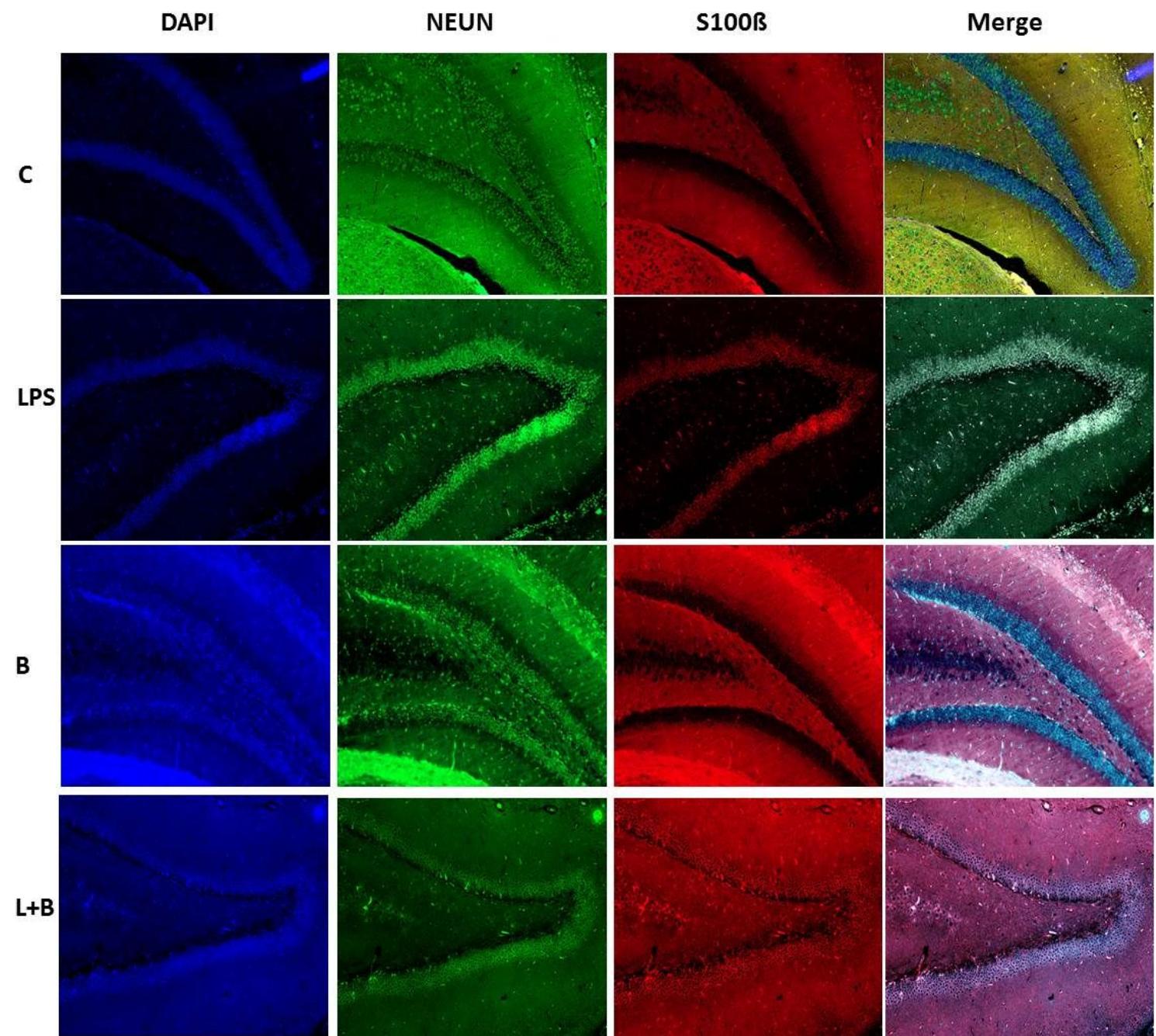


Figure 3

Brain tissue sections were stained experimental groups with DAPI, NEUN, S100 β antibodies x 10

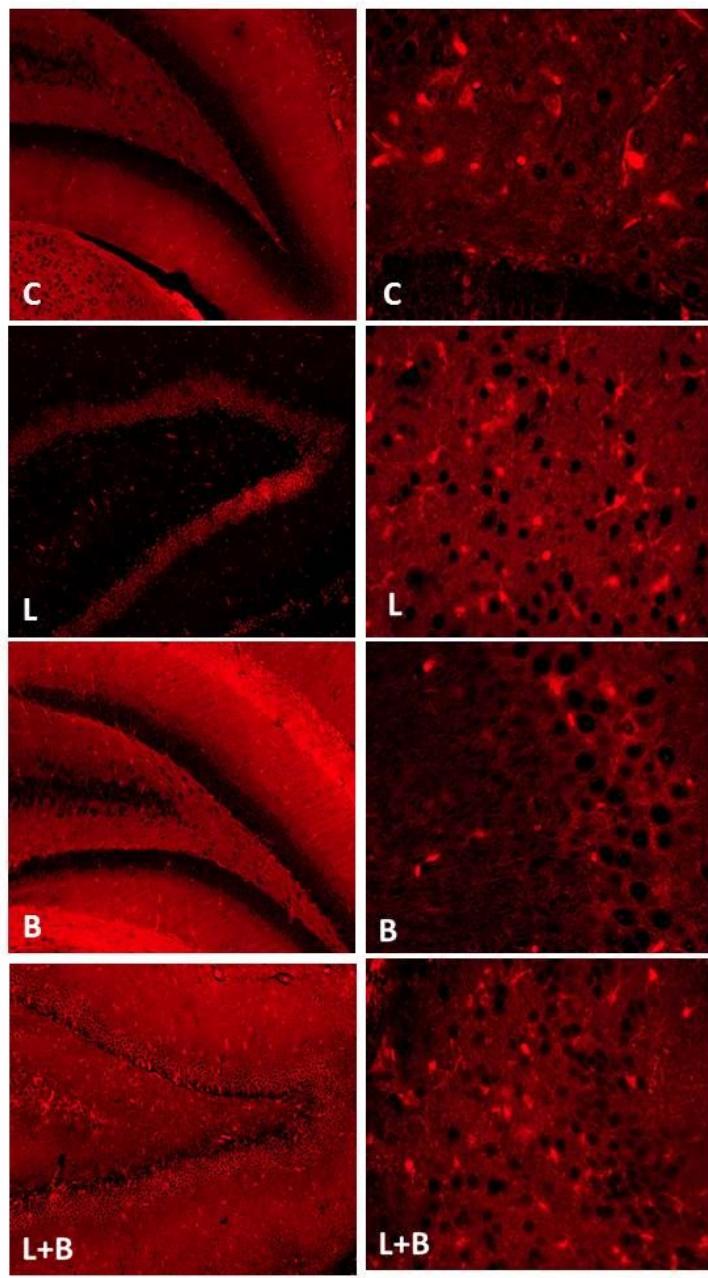


Figure 4

Brain tissue sections were stained experimental groups with S100 β antibody x 40

Figure 5

Brain tissue sections were stained experimental groups with DAPI, NEUN, Synaptophysin antibodies x 10

Figure 6

Brain tissue sections were stained experimental groups with Synaptophysin antibody x 40

Figure 7

Scores of brain tissue sections were stained experimental groups with DAPI, NEUN, s100 β antibodies

Figure 8

Scores of brain tissue sections were stained experimental groups with Synaptophysin antibody