Asia Pacific Academy of Science Pte. Ltd.

Journal of Biological Regulators and Homeostatic Agents 2026, 40(1), 3807.
https://doi.org/10.54517/jbrha3807

Article

Selective $GABA_B$ receptor antagonist, CGP-55845, promotes remyelination and oligodendrocyte diferentiation in rats with experimental autoimmune encephalomyelitis (EAE)

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CITATION

Zamri NA, Shafin N, Zakaria R, et al. Selective $GABA_B$ receptor antagonist, CGP-55845, promotes remyelination and oligodendrocyte diferentiation in rats with experimental autoimmune encephalomyelitis (EAE). Journal of Biological Regulators and Homeostatic Agents. 2026; 40(1): 3807. https://doi.org/10.54517/jbrha3807

ARTICLE INFO

Received: 24 June 2025
Revised: 10 July 2025
Accepted: 26 August 2025
Available online: 16 January 2025

Abstract: **Background**: Multiple sclerosis (MS) is an immune-mediated inflammatory disease of the central nervous system characterized by demyelination and axonal damage. This chronic neurological disorder afects millions worldwide and poses a significant economic and social burden. Current therapeutic strategies for MS primarily focus on mitigating neuroinflammation but do not efectively reverse demyelination. The $GABA_B$ receptor is expressed in oligodendrocytes and immune cells. $GABA_B$ receptors appear to play a role in modulating immune cells and promoting remyelination of damaged neurons. However, the efects of $GABA_B$-receptor modulation in animal models of MS remain largely unexplored. Therefore, this study evaluates the therapeutic role of $GABA_B$ receptor activity in rats with experimental autoimmune encephalomyelitis (EAE) to mimic MS in humans. **Methods**: EAE was induced in rats by immunization with 200 μg myelin oligodendrocyte glycoprotein (35-55) peptide in complete Freund's adjuvant containing killed Mycobacterium tuberculosis. Additionally, rats received injections of 200 ng pertussis toxin on the day of immunization and 48 h later. The EAE rats were treated with normal saline, CGP-55845, baclofen, or CGP-55845 + baclofen, i.p. Myelination in the lumbar spinal cord was assessed in control rats at 18- and 35-day postinduction. **Results**: CGP treatment significantly enhanced remyelination in EAE rats. This was evidenced by significant improvements in body weight and EAE clinical scores, as well as favorable histological changes. The observed increase in myelin expression in CGP-treated animals suggests that CGP-55845 promotes both remyelination and oligodendrocyte diferentiation. **Conclusions**: Our results suggested that the selective $GABA_B$ receptor antagonist CGP-55845 plays a significant role in promoting neural stem cell proliferation, particularly oligodendrocyte cells and remyelination in the EAE model of MS.

Keywords: GABA-B receptor antagonists; encephalomyelitis; autoimmune; experimental; receptors; GABA-B; multiple sclerosis; remyelination

1. Introduction

The immune-mediated disease known as multiple sclerosis (MS) induces central nervous system (CNS) damage and demyelination through inflammation triggered by an abnormal host immune response [1]. MS afects an estimated 2.8 million people globally, or 35.9/100,000 [2]. There are persistent gaps in the prevalence estimates, despite an increase in MS prevalence worldwide since 2013 [3]. The overall incidence rate among the 75 reporting nations is 2.1/100,000 people/year, with a mean age of 32

years at diagnosis. Research by Makhani et al. reviewed MS incidence and prevalence across multiple countries from 1985 to 2011, finding the highest rates inAustralia and the lowest in Africa [4,5]. MS prevalence increased over time, ranging from 0.67/100,000 in Taiwan to 3.67/100,000 in Australia, with black South Africans having the lowest (0.22/100,000) and Australian-born individuals the highest (125/100,000) [5].

MS is a chronic, immune-mediated, inflammatory disease of the CNS characterized by the infiltration of immune cells, including phagocytic and T lymphocytes, leading to demyelination and neurodegeneration. This process disrupts nerve signal transmission due to the degradation of neuronal material and the release of inflammatory factors, resulting in the formation of plaques and lesions within the CNS. Consequently, individuals with MS may experience a range of neurological deficits, such as visual disturbances, incoordination, fatigue, and mental disorders [6]. Efective strategies to reduce the prevalence and impact of MS are critical for addressing future clinical and public health needs.

The experimental autoimmune encephalomyelitis (EAE) model is a widely used preclinical model for elucidating the pathogenic mechanisms of MS [7]. This model is induced through immunization with myelin-derived antigens, such as MOG and myelin basic protein (MBP), which elicit an autoimmune response characterized by T- and B-lymphocyte reactivity against myelin, and subsequent inflammatory responses leading to myelin sheath damage [8]. B cells, through the production of antimyelin antibodies, contribute to thepathogenesis, and macrophages and microglia mediate myelin destruction, culminating in the formation of characteristic plaques and lesions [9]. Although remyelination can occur, iterative cycles of demyelination may ultimately result in irreversible neurodegeneration, replicating key pathological features of MS [10].

Currently, the majority of FDA-approved pharmaceutical interventions for MS consist of immunosuppressive drugs, often referred to as disease-modifying therapies. These agents primarily target and reduce inflammatory components to reverse the progression of the disease. However, existing treatments do not address the progressive neuronal dysfunction and disability associated with MS [11]. Although immunosuppression can efectively prevent or delay MS exacerbations, it does not guarantee lifelong protection from relapses. Furthermore, the long-term tolerability of current therapies remains a concern, as chronic immunosuppressive drug use can increase a patient's susceptibility to infection and impose a significant financial burden [12].

Little is known about the role of $GABA_B$ receptors in remyelination, but they are widely expressed in the spinal cord [13] and may have efects on the neurogenic niche within this region. The proliferative activity of neural stem cells (NSCs) in the spinal cord is limited; studies suggest that NSCs ofthe central canal can be prompted to proliferate by a variety of methods including modulation of neurotransmission within the neurogenic niche. The efects of increasing the proliferation of NSCs and their precursor stimulate the myelination capability of a neuron. Much research has shown positive modulation of the oligodendrocytes in shaping the myelination capability in normal and demyelinating diseases. Several studies provide evidence for the influence of $GABA_B$ receptors on neurogenic niches in the brain [14] and a study on brainstem cerebrospinal-fluid-contacting cells showed $GABA_B$ receptor-mediated

efects on these cells [14]. Oligodendrocytes, the cells responsible for myelination, arise from ependymal cells [15], a key component of neural stem cell populations, able to increase the neurogenic activity in the spinal cord and may enhance remyelination. Baclofen (a selective $GABA_B$ receptor agonist) administration also hyperpolarizes ependymal cells [16,17], an efect antagonized by CGP 55845 ($GABA_B$ antagonist) [18], indicating a further role for $GABA_B$ receptors on the neurogenic niche. A study by Giachino et al. used $GABA_B$ genetically-deleted mice $GABA_{B1}$-/- or $GABA_{B2}$-/- to show that impairing $GABA_B$ signaling pharmacologically or in mice deficient for the $GABA_{B1}$ receptor gene, increased cell proliferation in the dentate gyrus, whereas activating $GABA_B$ receptors had the opposite efect [19].

Remyelination holds promise as a therapeutic approach, as it enables neurons to restore their signaling capabilities, potentially mitigating MS-related deficits [13]. A significant unresolved challenge is that there are presently no therapies available for the progressive phases of MS. Long-term demyelination is now believed to play a role in the neurodegeneration that appears in the later stages of MS [14]. Emerging research suggests that $GABA_B$ receptors, expressed in oligodendrocytes and immune cells, play a role in promoting remyelination and modulating inflammatory responses [19,20].

In regard to the mechanism that can promote remyelination, particularly in the context of MS and experimental models of demyelination, cannabinoid-based treatments showed the ability to promote oligodendrocyte survival through anti-inflammatory efects, oxidative stress reduction, and apoptosis inhibition as reviewed in ref. [21]. As reviewed in ref. [21], cannabinoids can support CNS remyelination through oligodendrocyte precursor cell diferentiation, enhancement of myelin production, and neuroprotection. Therefore, promoting remyelination is a key strategy aimed at preventing permanent neurological disability, and clinical trials are currently exploring drugs that can enhance oligodendrocyte diferentiation. Previous studies have suggested that $GABA_B$ receptor activation promotes oligodendrocyte progenitor cell (OPC) diferentiation, leading to enhanced remyelination [22]. $GABA_B$ receptor agonists like baclofen have been reported to enhance myelin protein production in lysolecithin-treated cerebellar slices and to improve OPC diferentiation in demyelinated spinal cord models [23]. Given that myelin remodeling requires a balance between demyelination and remyelination, sustained $GABA_B$ receptor activation can contribute to demyelination by altering the natural turnover of oligodendrocytes or interfering with their survival [23].

Our study explored the potential of $GABA_B$ receptor signaling as a therapeutic target for MS. Specifically, this research investigated the impact of $GABA_B$ receptor modulation on remyelination in an EAE rat model. We hypothesized that such modulation positively influences both remyelination and behavioral outcomes in the model.

2. Materials and methods

2.1. Experimental animals

Sixty male Sprague-Dawley rats (weighing 100–200 g, aged 4–8 weeks old) were housed individually in the colony room with a constant temperature of 22 ± 1.0 ˚C, humidity 45–65% and a 12–12 h light-dark cycle; lights on at 7 a.m. and of at 7

p.m. The rats were fed with standard food pellets and water *ad libitum.* The sample size for each group of the rats (n = 6) was identified by using G * power version 3.0.10 software with α (type-1 error) of 0.05, a power of 0.8, an efect size of 0.85, which was derived from comparable outcomes in a prior study [24]. An additional 20% drop-out based on a previous study was included to ensure adequate statistical power despite possible dropouts. The protocols of this study were designed to minimize animal sufering and were approved by the USM Institutional Animal Care and Use Committee, Universiti Sains Malaysia, Malaysia (USM IACUC) [USM/ IACUC/2021/ (127) (1134)]. Instructions on animal handling and care were strictly followed according to the Guidelines for the Care and Use of Laboratory Animals by the National Institute of Health. Every attempt was made to minimize the number of animals used and the sufering they endured.

2.2. Induction of EAE

EAE was induced by immunization (s.c.) with 200 μg MOG (35–55) (AnaSpec, Cat# AS-60130-1, USA) peptide in CFA (Sigma-Aldrich, Cat# F5881, USA) containing 2–5 mg killed mycobacterium tuberculosis H37Ra/mL emulsion. Rats were also injected i.p. with pertussis toxin (200 ng) (Sigma-Aldrich, Cat# 70323-44-3, USA), diluted in PBS on the day of immunization and 48 h later. The doses of MOG (35–55) peptide (200 μg) and pertussis toxin (200 ng) were selected based on the published protocol [25]. The inclusion of pertussis toxin on the day of immunization and 48 h later ensured optimal disruption of the blood–brain barrier, a critical step in EAE pathogenesis.

2.3. Groups of immunized rats

After immunization, the rats were randomly divided into five groups (n = 6/gp), and n = 30 for each experimental phase (18 and 35 days): control (Healthy) group; normal saline as a vehicle (NS) group; CGP-55845 (CGP) group; baclofen (Bac) group; and CGP-55845 + baclofen (CGP + Bac) group. The study was implemented over two experimental durations, 18 days and 35 days to assess the time-dependent efects of CGP 55845, Baclofen and their combination in the context of MOG-induced EAE in rats. To reduce observer bias during behavioral assessments, scoring was performed by independent evaluators who were not involved in treatment administration. Additionally, diferent evaluators were rotated on a daily basis to avoid scoring familiarity and to ensure consistency.

2.4. $GABA_B$ receptor administration

CGP-55845 (10 μg/mL) [26], a $GABA_B$ receptor antagonist (Sigma-Aldrich, USA) was dissolved in DMSO: 5 mg/mL and diluted with normal saline. Next, baclofen (2 mM) [23], a $GABA_B$ receptor agonist (Alfa Aesar, USA) was diluted with normal saline. Both reconstituted solutions for injections were stored at 4 °C. The CGP-55845 + baclofen was the combination of both drugs after dilution. For each day of injection, each rat received i.p injection at a similar time during a period of light (0900 h). The i.p. injection of these substances was administered for

0.5 mL/injection each day, continuing until the treatment was considered clinically relevant. The doses and rationale of the drugs followed findings from Serrano-Regal et al. [16], which suggested that baclofen may enhance remyelination by promoting oligodendrocyte progenitor cell diferentiation, indicating its potential therapeutic application in demyelinating diseases like MS [16]. Serrano-Regal et al. [16] demonstrated that these concentrations efectively modulated GABAB receptor activity in vivo, leading to enhanced OPC diferentiation and promoting remyelination. These doses were chosen to replicate the observed therapeutics efects relevant to demyelinating conditions. Studies have shown that CGP-55845 efectively blocks both pre-and post-synaptic $GABA_B$ receptors, making it a valuable tool for investigating $GABA_B$ receptor functions in various neurological contexts [15]. The combination therapy was used to show the balance between inhibitory and excitatory signals in the CNS and their implications in neuroinflammatory and neurodegenerative conditions.

2.5. Daily body-weight assessment

Each rats' body weight was measured daily from Day 1 until Day 35. The data are expressed in daily body weight and percentage of change in body weight relative to the baseline weight (Day 1) as shown in the formula below:

$$\% \text{ Change of body weight (BW)} = \frac{\text{BwonDayN-BwonDayI}}{\text{BwonDayI}}$$

In which "N" refers to each day the body weight was measured. Both absolute and percentage weight change were measured to provide a comprehensive assessment of disease progression and treatment response. Absolute weight allows for direct comparisons at each time point, while percentage change shows individual rats' baseline diferences. This dual approach showed the accuracy of weight-related outcome measurements in the EAE model.

2.6. Daily EAE scoring

The behavior assessment consisted of the severity of EAE which was scored daily using a grading scale of 0–5, following the published protocol [27]: 0 = unafected; 0.5 = partially limp tail; 1 = paralyzed tail; 2 = hind limb paresis and loss in coordinated movement; 2.5 = one hind limb paralyzed; 3 = both hind limbs paralyzed; 3.5 = hind limbs paralyzed and weakness in forelimbs; 4 = forelimbs paralyzed; and 5 = moribund/death. To further prevent inter-observer bias, the assessment of the EAE score was carried out by two individuals who were blind to the treatment groups.

2.7. Protocol and euthanasia of rats

After the induction of EAE, the rats were measured daily for weight and EAE scores. The rats were divided into 5 groups as mentioned above. All rats were injected i.p. The %-ethynyl-2'-deoxyuridine EdU injection started 3 days before euthanasia. Rats were euthanized at 18 and 35 days postimmunization by an overdose of sodium pentobarbitone (> 60 mg/kg, i.p.). Then, the spine was collected by laminectomy. All other nonutilized tissues and body parts were removed, and the carcasses were disposed of properly. The protocols for all 5 groups are illustrated in **Figure 1.**

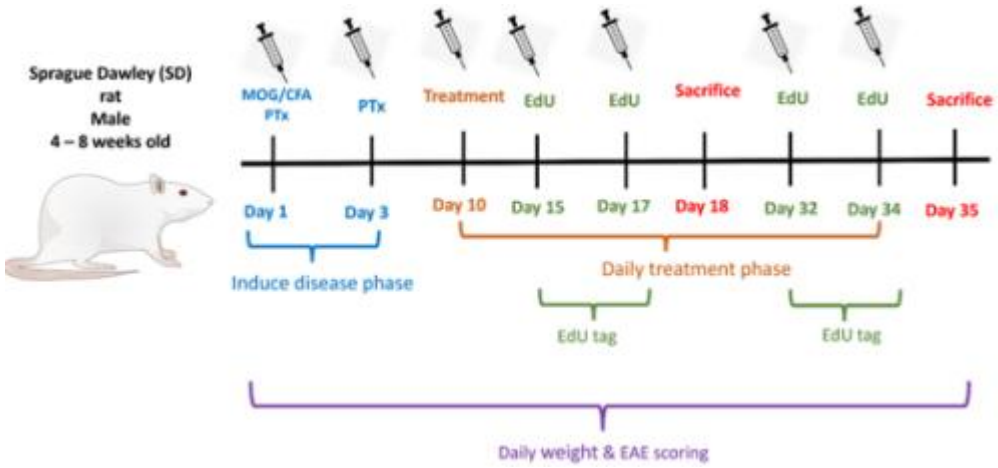

Figure 1. Protocol for rat groups: normal saline (NS); CGP-55845 (CGP); baclofen (Bac); and CGP-55845 + baclofen (CGP + Bac) groups from Day 1 until Day 35.

2.8. Tissue processing and histology

Spinal cords were collected and prepared as previously described [25]. After the sodium pentobarbitone exposure, the mobility ofthe rat was observed, indicating its consciousness and any sign of motor impairment. The rat's paw was pinched; if no sign of reflex was present (which meant that the rat was deeply anaesthetized), the rat was transcardially perfused with 0.1 M phosphate bufer (PB) and fixed with 4% paraformaldehyde in PB. After laminectomy, the spinal cord was kept in the 4% PFA for 24 h and then transferred to PB and stored at 4 °C.

For each rat, a lumbar spinal cord segment (L1–L4) of about 8 mm was isolated and processed. For paraffin embedding, spinal-cord-tissue samples were fixed with 4% PFA for 24 h. They were then dehydrated in 95% ethanol for 3 h, 100% ethanol for 4 h, xylene for 3 h, and paraffin for 3 h. Tissue samples were then embedded in paraffin and sectioned (5–10 μm thick) using a microtome. The paraffin-embedded samples were stained with a LFB Stain Kit (Myelin Stain) (Abcam, UK) (ab150675) to examine myelination. The spinal cord sections were deparaffinized, incubated for 2 h at 60 ºC, rinsed in water, dipped into lithium carbonate solution and alcohol reagent for diferentiation and retention of blue staining, rinsed in water, dipped into cresyl echt violet to highlight neuronal cell bodies, rinsed in water to remove excess stain, dehydrated in absolute alcohol, and mounted in glass slides.

2.9. EdU click chemistry

Spinal cord slices that were fixed with 4% paraformaldehyde in 0.1 M phosphate-bufered saline (PBS) were rinsed with PBS with 0.2% Triton 100 (PBS-T) for 20 min. Then, the slices were washed with 0.1 M Tris Bufer (pH 7.6) twice for 10 min. Then, dH_2O, 2.0 M Tris Bufer (pH 8.5), 10 mM copper (II) sulphate, 1 mM biotinylated azide, and 0.5 M ascorbic acid were added and then agitated for 30 min in the dark. The slices were rinsed with 0.1 M Tris Bufer (pH 7.6) twice for 10 min and washed with 0.1 M PBS for 10 min. After incubation, the EdU was detected with a secondary antibody (Streptavidin 555), and agitated for 2 h at room temperature. Cells were washed three times in 0.1 M PBS. This method covalently binds an azide

to an alkyne. It utilizes a copper catalyst and an azide-modified fluorescent dye to form a stable triazole ring that can be seen under a fluorescent microscope [28].

2.10. Immunohistochemistry

After successfully viewing EdU, the spinal cord slices were washed with 0.1 M PBS for 10 min. Then, the slices were blocked with 10% goat serum in PBS for 30 min, then incubated with primary antibody anti-Olig2 (bs-11194R, 1:500, Abcam) in PBS with 0.2% Triton 100 (PBS-T) overnight at 4 °C. After incubation, primary antibodies were detected with secondary antibodies: Alexa Fluor 488 (ab150113, 1:1000, Abcam) for 1 h at room temperature. The slices were mounted on glass slides with fluorescent mounting medium with DAPI to stain cell nuclei (Vectashield).

2.11. Histological evaluation

A light microscope (BX41-32P02, Olympus) was used to examine the sections. For each rat, equal intervals were selected for quantification. In the case of the lumbar section, the areas of interest in 4× and 10× power fields were analyzed and averaged. The same microscope with fluorescence filters was used to view EdU and Olig2 counts on the spinal cord slices. The ImageJ (NIH, Bethesda, MD) was used for quantitative analysis of demyelinating plaque, LFB color intensity of the lumbar spinal cord, and EdU and Olig2 cell counts. The myelin quantification method (MQM) was used, as above, to obtain more precise estimates of demyelination and myelin intensity [29]. Sections were mounted on glass slides and examined for myelin and demyelination. This was performed by highlighting myelin (blue) and demyelinated areas (pale or unstained) in the LFB-stained sections. Plaques were quantified by measuring the area of density using ImageJ analysis software. The region of interest (ROI) was chosen according to the position of the spinal cord slices. The slices were initially marked and divided as anterior and posterior, and further divided into left and right. We could not determine the exact size and shape of the ROI since we were focusing on the general efects of EAE and drug modulation. The slice was placed at the same distance from the midline in each section and comparisons across experimental groups were done.

2.12. Western blotting

The lumbar region of the spinal cord was quickly removed and snap frozen at –80 °C. The tissues were harvested for Western blot analysis (Western Blot Detection Kit, Elabscience) according to the standard protocol as presented in the manufacturer's instructions. The tissues were homogenized in lysis bufer and the supernatant. Protein concentrations in the extracts were determined using the kit. Ten μg of protein per lane from the protein extracts were separated on a 12% SDS-PAGE gel (BioRad, USA) and transferred to PVDF membranes using an electrophoretic transfer system (Cleaver Scientific, UK). The blots were washed, and nonspecific binding sites were blocked with 5% TBS-T with skimmed milk using the kit. Then, the blots were incubated overnight at 4 °C with the following primary antibodies: anti-$GABA_{BR1}$ [EPR22954-47 (ab238130), 1:500, Abcam, UK], and anti-β-actin (sc-47778, 1:500, Santa Cruz, USA). The secondary antibodies were goat antirabbit IgG H&L (HRP) (E-AB-1003, 1:1000, Abcam, UK) for 1 h at room temperature. Blots

were visualized using enhanced chemiluminescence (ECL, Western Blot Detection Kit, Elabscience) and scanned using a luminescent image analyzer. The results were visualized using an enhanced chemiluminescence system. Densitometric analysis was conducted using ImageJ software (NIH, USA). The levels of analyzed proteins were normalized for β-actin protein.

2.13. Statistical analysis

All data were first assessed for normality by histogram analysis and Shapiro-Wilk tests. For the longitudinal data, including daily body weight, and daily EAE score, repeated measures analyses of variance (ANOVA) was conducted to account for within-subjects variations over time. For cross-sectional comparisons at Day 18 and Day 35 time points, one-way ANOVA was used to analyze the quantitative demyelinating plaque of the lumbar section of spinal cords, the expression of blue intensity of LFB, the EdU cell count, the Olig2 cell count, and the mean relative $GABA_{BR1}$ subunit protein level. Tukey's post hoc test was used for comparisons. Mixed-design ANOVA was used to analyze the association between control, 18-day, and 35-day time points in the EAE model, followed by Tukey's post-hoc test used for pairwise comparisons. All data were presented as mean ± standard deviation (SD) and analyzed using GraphPad Prism version 9 software (Dotmatics, United States). A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Successful induction of EAE model

3.1.1. Decrease in body weight following MOG-induced EAE

The Healthy group served as the baseline reference for assessing weight gain. The group showed a consistent upward trend in weight over both the 18- and 35-day observation periods, reflecting normal and healthy growth patterns (**Figure 2**). In

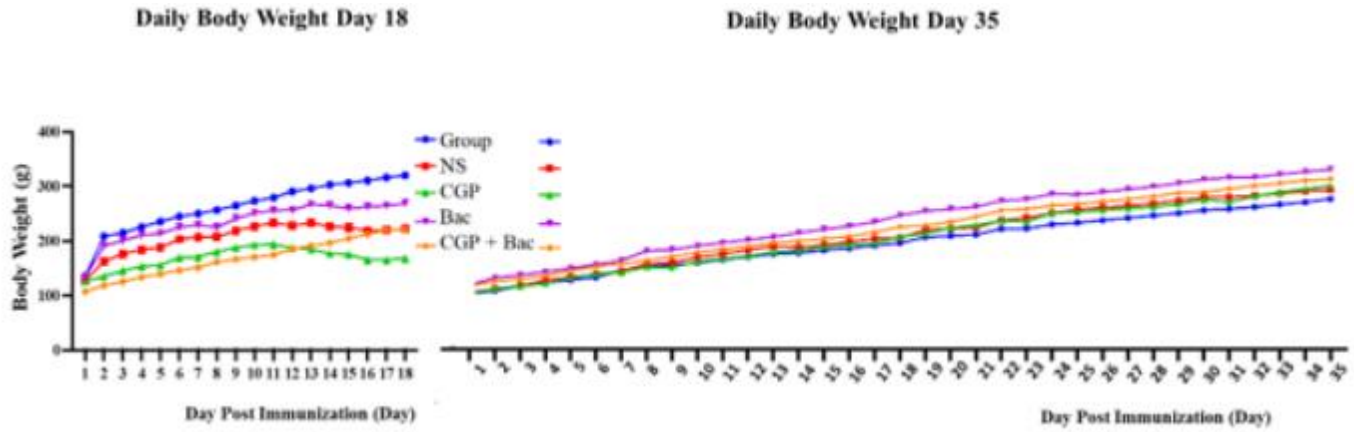

Figure 2. Mean daily body weight between 18 and 35 days for all experimental groups. A transient reduction in body weight was observed in the CGP and NS groups during the initial 18 days of EAE induction. However, all groups demonstrated a consistent and progressive increase in body weight during the subsequent 35-day period, indicating recovery and normal growth patterns. Asterisks (*) denote statistically significant diferences ($p < 0.05$) between the experimental groups and the healthy control group.

contrast, groups undergoing therapeutic modulations initially exhibit minimal weight changes during the first 18 days. However, a marked and sustained recovery is observed in these groups by day 35 of treatment, suggesting that interventions gradually enabled the promotion of the normal trends of growth.

Rats treated with normal saline (NS) and baclofen (Bac) showed substantial body weight loss and were significantly diferent from the Healthy group, suggesting that the active progression of the disease influenced the observed changes in body weight. Tukey's post hoc multiple comparisons for 18 days were statistically significant ($p < 0.0001$), suggesting substantial weight changes due to treatment and for 35 days, all group comparisons showed significant diferences ($p < 0.0001$) indicating that weight changes remained persistent over time. There is significant weight loss in the NS group compared to the healthy group confirming that EAE induction impacts body weight. And the comparison between NS and CGP for the 18-day group showed that CGP treated rats showed significantly less weight loss than NS, indicating a protective efect ofCGP (**Table 1**).

Efects of treatment on body weight in EAE-induced rats at Day 18 and Day 35 postimmunization. Statistical comparisons were performed using repeated measure ANOVA followed by Tukey's post hoc test. Significant diferences were observed among groups on both Day 18 and Day 35 (*$p < 0.0001$ was considered significant), indicating treatment-related changes in body weight over time. Abbreviations: NS = Normal Saline; Bac = Baclofen; CGP = CGP-55845.

3.2. Disease severity based on EAE score progression

The EAE score for the Healthy group remained at 0 throughout the study, indicating no disease progression. In contrast, the EAE groups (NS, CGP, Bac, and CGP + Bac) exhibited a consistent increase in EAE scores over time, peaking at around Day 12–13, reflecting the progression of disease symptoms. By Day 18, a leveling of in the EAE scores was observed in these groups which persisted until Day 35. This plateau suggests that postinduction with $GABA_B$ modulation helps stabilize the disease and prevents further progression of symptoms. In the case of the CGP55845 (CGP) group, this led to a faster and more gradual stabilization of EAE scores than in other modulation groups (**Figure 3**).

Table 1. Tukey's post-hoc multiple comparisons for daily body weight for 18 and 35 days of induction.

Group comparison	Mean diference (18 days)	Mean diference (35 days)
Healthy vs. NS	57.02*	–13.04*
Healthy vs. CGP	94.27*	–9.64*
Healthy vs. Bac	28.62*	–40.50*
Healthy vs. CGP + Bac	94.89*	–25.57*
NS vs. CGP	37.25*	3.40*
NS vs. Bac	–28.40*	–27.46*
NS vs. CGP + Bac	37.87*	–12.53*
CGP vs. Bac	–65.65*	–30.86*
CGP vs. CGP + Bac	0.616	–15.93*
Bac vs. CGP + Bac	66.27*	14.93*
F and*p* values	$F_{17,68} = 21.88$ $p < 0.0001$	$F_{34,136} = 576.8$ $p < 0.0001$

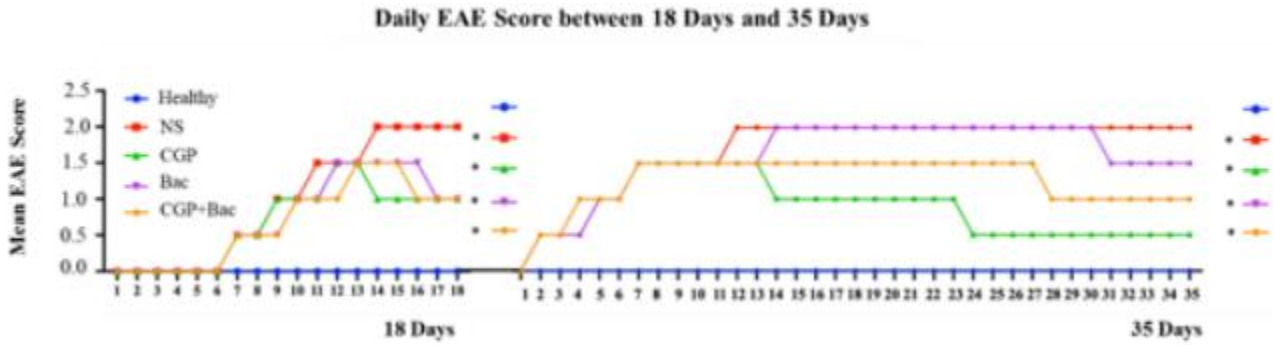

Figure 3. Mean EAE scores between 18 and 35 days for all experimental groups. The CGP group exhibited a significantly reduced EAE score compared to other groups on both the 18th and 35th days, reflecting its therapeutic impact. Asterisks (*) denote statistically significant diferences ($p < 0.05$) between the experimental groups and the healthy control group.

The results of the post-hoc repeated measures ANOVA conducted on the daily EAE score for both 18 and 35 days in the NS group showed significantly higher EAE scores compared to healthy controls (**Table 2**). This confirmed the expected increase in disease severity, i.e., EAE-induced. The increasing diference of 35 days suggests disease progression over time. In the CGP group, for 35 days of induction, CGP significantly reduced EAE scores compared to NS, supporting its neuroprotective efects. Baclofen-treated rats showed significant improvement but were still worse than CGP-treated animals shown by Healthy vs. Bac (–0.667, $p < 0.0001$ at 18 days; –0.857 at 35 days). The combination group showed significant improvement compared to the NS group confirming a treatment efect shown by NS vs. CGP + Bac (0.306 at 18 days, 0.443 at 35 days, $p < 0.0001$). CGP alone had a slight advantage over the combination therapy; CGP vs. CGP + Bac (0.000 at 18 days; –0.386 at 35 days, $p < 0.0001$). Baclofen-treated rats had slightly higher EAE scores than CGP + Bac, confirming that the combination was better than Baclofen alone.

Mean diferences in daily EAE score in EAE-induced rats at Day 18 and Day 35 postimmunization. Statistical comparisons were performed using repeated

Table 2. Tukey's post-hoc multiple comparisons for daily EAE score for 18 and 35 days of induction.

Group comparison	Mean diference (18 days)	Mean diference (35 days)
Healthy vs. NS	0.972*	–1.686*
Healthy vs. CGP	–0.667*	–0.857*
Healthy vs. Bac	–0.722*	–1.586*
Healthy vs. CGP + Bac	–0.667*	–1.243*
NS vs. CGP	0.306	0.829*
NS vs. Bac	0.250	0.100*
NS vs. CGP + Bac	0.306*	0.443*
CGP vs. Bac	–0.056	–0.729*
CGP vs. CGP + Bac	0.000	–0.386*
Bac vs. CGP + Bac	0.056	0.343*
F and *p* values	$F_{17,68} = 10.67$ $p < 0.0001$	$F_{34,136} = 4.711$ $p < 0.0001$

measure ANOVA followed by Tukey's post hoc test. Significant diferences were observed among groups on both Day 18 and Day 35 (*$p < 0.0001$ was considered significant), in all groups of 35 days of induction indicating treatment-related changes in EAE score over time. Abbreviations: NS = Normal Saline; Bac = Baclofen; CGP = CGP-55845. *$p < 0.05$ was considered significant. Abbreviations: NS = Normal Saline; Bac = Baclofen; CGP = CGP-55845.

3.3. LFB analysis of demyelination in lumbar spinal cord

The histological analysis of the LFB staining suggested a more disseminated pattern of demyelinating plaques in the spinal cord, reminiscent of MS plaques, because the rats were immunized with MOG in CFA. The results of induction of MOG and CFA caused areas of myelin loss in tissue samples across groups (NS, CGP, Bac, and CGP + Bac) (**Figure 4**). Comparing the control (Healthy) group to experimental groups helped determine how the condition or modulation afected myelin integrity over time. Quantitative analysis of demyelinating plaques (**Figure 4**) showed a statistically significant diference among groups at both 18 and 35 days postinduction. On day 18, one-way ANOVA revealed that all MOG + CFA-induced groups, namely NS (Normal Saline), CGP, Bac, and CGP + Bac, had significantly increased demyelinating plaque areas compared to the healthy control ($p < 0.05$). Among the treatment groups, CGP

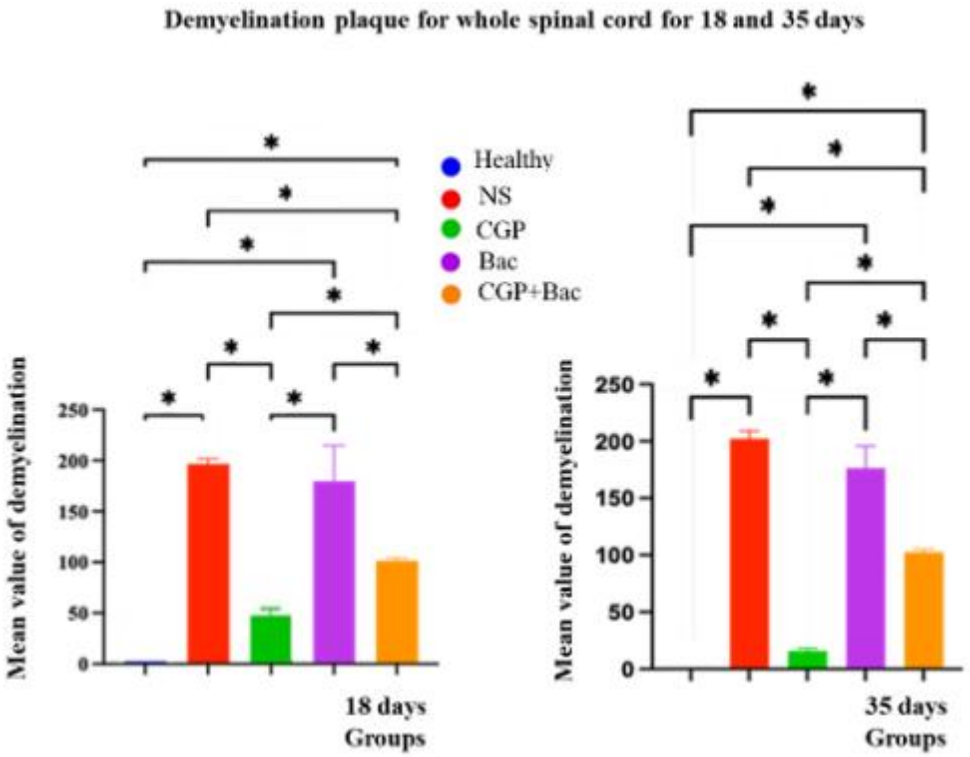

Figure 4. Mean relative demyelinating plaque for the whole spinal cord between 18 and 35 days for all experimental groups. The CGP group exhibited a significantly reduced demyelinating plaque compared to other groups on both the 18th and 35th days, reflecting its therapeutic impact. The comparison between NS and CGP groups showed significant diferences, indicating that CGP treatment improved the demyelination in the spinal cord. Asterisks (*) denote statistically significant diferences ($p < 0.05$) between the experimental groups, where mean ± standard deviation (SD).

and CGP + Bac showed a marked reduction in plaque size relative to the untreated NS group, suggesting early therapeutic efects. By day 35, the trend continued with significant diferences observed across all groups ($p < 0.05$). The CGP + Bac combination group exhibited the most substantial reduction in demyelinating area, indicating a potential synergistic efect of the combined therapy in preserving myelin integrity. These results support the efectiveness of the treatments in modulating demyelination in this EAE model over time.

Figure 5 illustrates the histological findings for Day 18, highlighting the early progression of demyelinating across the groups. In the Healthy group, there was no significant plaque appearance. The extensive demyelinating plaque was observed in the NS group, mild and moderate in the Bac group, and the CGP + Bac group, respectively. In contrast, the remyelination occurred in the CGP group. **Figure 6** illustrates the histological findings of all groups for Day 35. The Healthy group demonstrated no significant plaque appearance. The extensive demyelinating plaque was observed in the NS group, mild and moderate in the Bac group and the CGP + Bac group, respectively.

To compare whether there were significant diferences in the mean number of demyelinating plaques among the groups, we used one-way ANOVA (**Table 3**). The results of the one-way ANOVA on the mean value of demyelinating plaque for Day 18 showed a statistically significant diference ($F_{4,10} = 94.84, p < 0.0001$). From Tukey's post-hoc multiple comparisons, the healthy vs. NS shows a highly significant increase in demyelination for both 18 days ($–196.6, p < 0.0001$) and 35 days ($–219.0, p < 0.0001$). This confirms EAE induction efectively triggers demyelination, validating the model. CGP treatment significantly reduced demyelination, indicating a strong neuroprotective efect. Baclofen alone showed a weaker efect in reducing demyelination, as it was not statistically significant for 18 days but became significant for 35 days ($16.96, p$ = ns for 18 days; $42.62, p < 0.0001$ for 35 days). CGP alone performed slightly better than CGP + Bac, suggesting that adding Baclofen did not enhance CGP's protective efects. In general, all NS comparisons at 35 days showed higher demyelination compared to 18 days, and this showed that EAE-induced demyelination worsens over time. Treatments (CGP, Bac, and CGP + Bac) continued to show significant diferences compared to NS, indicating that their protective efects were sustained over time.

Mean diferences in demyelinating plaques in the whole spinal cord were compared between groups using one-way ANOVA. Significant diferences were observed among groups on both Day 18 and Day 35 ($*p < 0.0001$ was considered significant), in all groups of 18 days except for NS vs. Bacandin all groups comparisons in 35 days of induction, indicating treatment-related changes in EAE score over time. $*p < 0.05$ was considered significant. Abbreviations: NS = Normal Saline; Bac = Baclofen; CGP = CGP-55845.

3.4. EdU-positive cell proliferation and oligodendrocyte lineage response in lumbar spinal cord

The purpose of EdU click chemistry is to stain newly proliferated cells. The EdU cell count analyzed the expression of newly proliferated cells from oligodendrocytes and evaluated the impact of $GABA_B$ receptor modulation on the expression levels. The graphical representation in **Figure 7** illustrates the EdU-positive cell counts across all

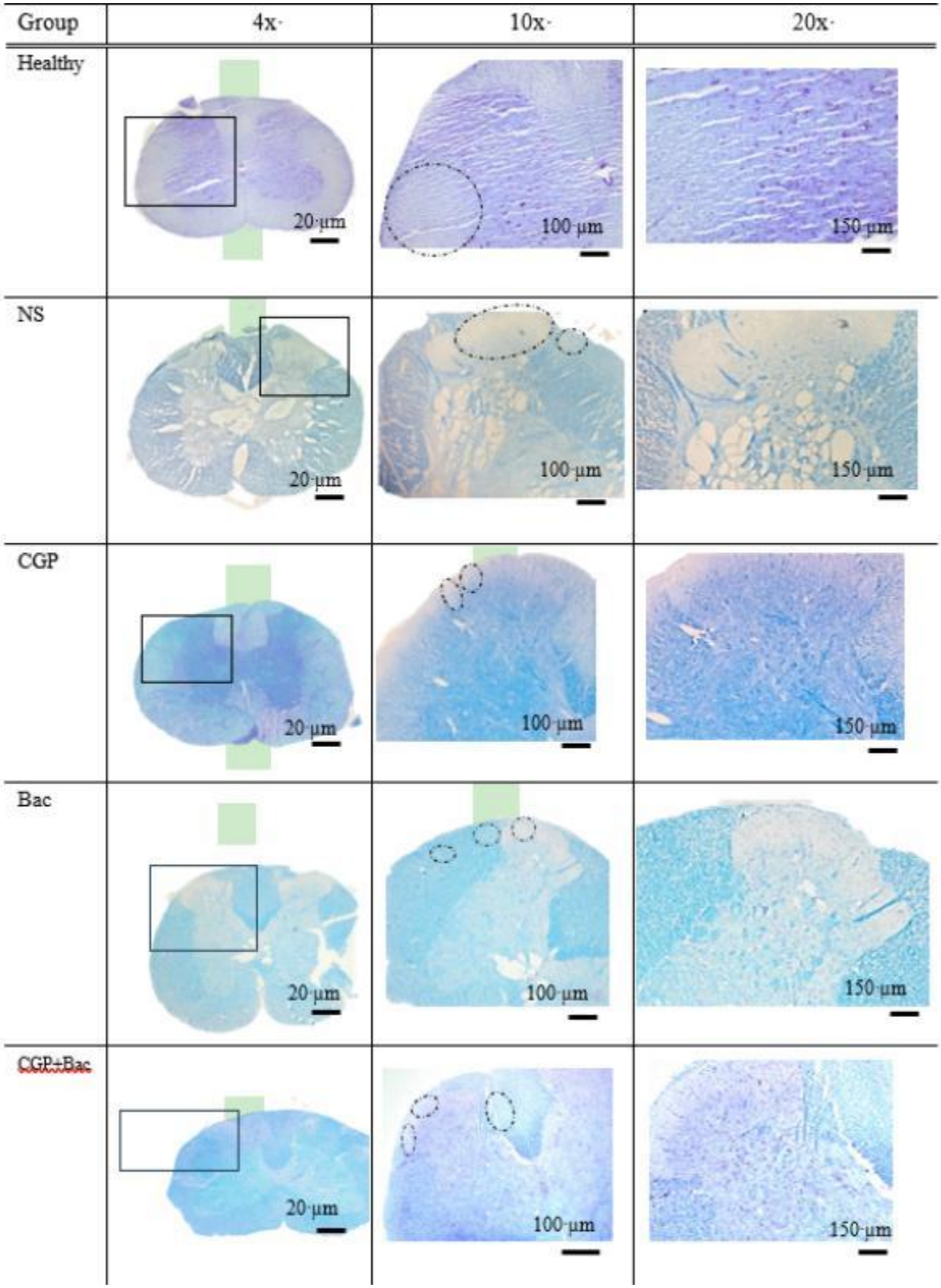

Figure 5. The demyelinating plaque of the spinal cord section for Day 18. The figure shows the histology section of the spinal cord of all groups from Day 18, subjected to LFB staining. Images are at 20, 100, and 150 µm scales. *The rectangle indicates which area is magnified. The circle indicates the demyelinating plaque on the spinal cord. The Healthy group demonstrated no significant plaque appearance. The remyelination occurred in the CGP group. The extensive demyelinating plaque was observed in the NS and Healthy groups, mild and moderate in the Bac group and CGP + Bac group.

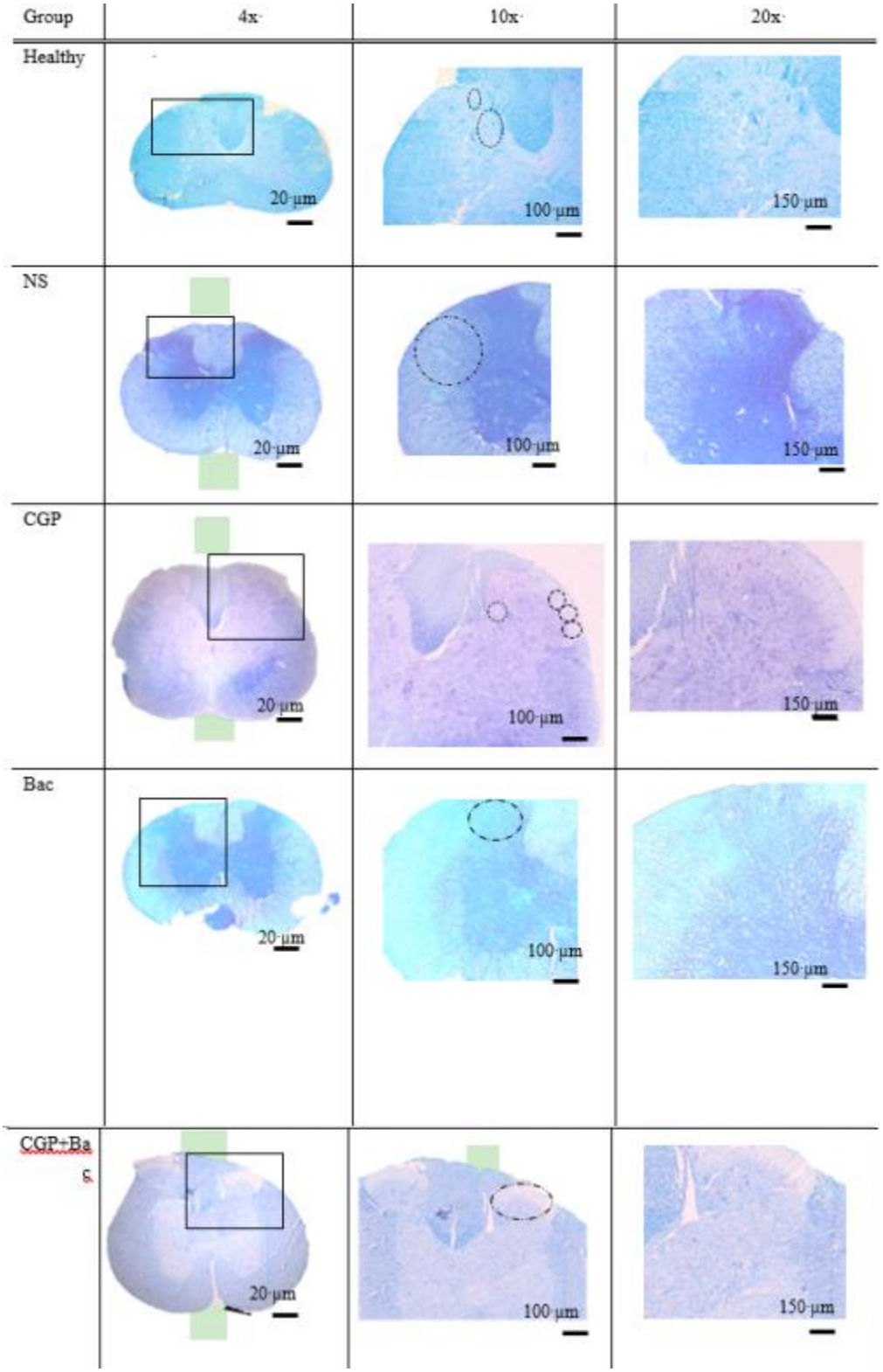

Figure 6. The demyelinating plaque of the spinal cord section for Day 35. The figure shows the histology section of the spinal cord of all groups from Day 18, subjected to LFB staining. Images are at 20, 100, and 150 µm scales. *The rectangle indicates which area is magnified. The circle indicates the demyelinating plaque on the spinal cord. The Healthy group demonstrated no significant plaque appearance. Meanwhile, the remyelination occurred in the CGP group. The extensive demyelinating plaque was observed in the NS and Healthy groups, mild and moderate in the Bac group and CGP + Bac group, respectively.

Table 3. Multiple comparisons for demyelinating plaques in the whole spinal cord for 18 and 35 days of induction.

Group comparison	Mean diference (18 days)	Mean diference (35 days)
Healthy vs. NS	–196.6*	–219.0*
Healthy vs. CGP	–17.68*	–16.01
Healthy vs. Bac	–179.7*	–176.3*
Healthy vs. CGP + Bac	–101.3*	–102.7*
NS vs. CGP	178.9*	202.9*
NS vs. Bac	16.96	42.62*
NS vs. CGP + Bac	95.29*	116.3*
CGP vs. Bac	–162.0*	–160.3*
CGP vs. CGP + Bac	–83.65*	–86.65*
Bac vs. CGP + Bac	78.33*	73.66*
F and *p* values	$F_{4,10} = 94.84$ $p < 0.0001$	$F_{4,10} = 11.6$ $p < 0.0001$

groups at Day 18. The CGP group shows a marked increase in cell proliferation compared to the Healthy and NS groups, consistent with the statistical findings. Similarly, the 35-day group shows the EdU cell counts at Day 35, where the CGP group again displays noticeably elevated cell proliferation compared to the Healthy, NS, and Baclofen groups. These visual trends align with the post-hoc analysis results reported in **Table 4**.

To determine whether there were diferences between multiple groups, we performed quantitative analysis of the between-group diferences. The results of the

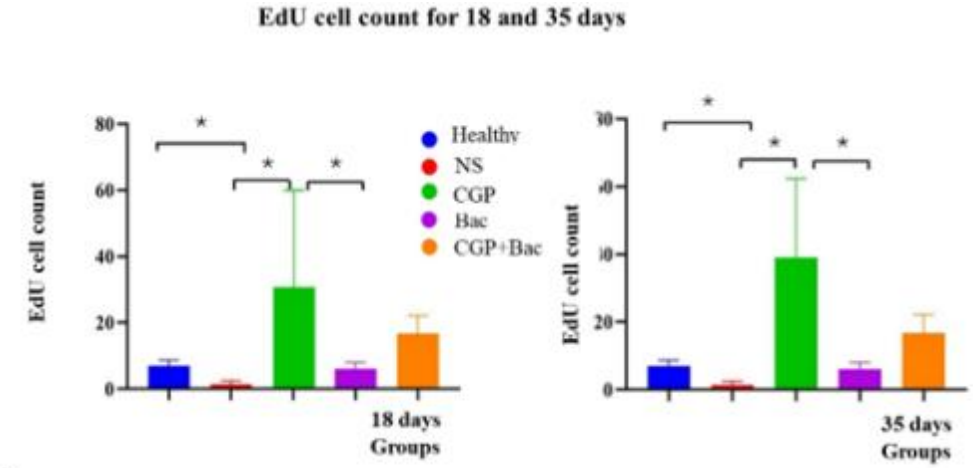

Figure 7. EdU cell count between 18 and 35 days for all experimental groups. EdU cell counts indicate the proliferative new-generating cells. The CGP group exhibited a significantly increased EdU cell count compared to other groups on both the 18th and 35th days, reflecting its therapeutic impact. The comparison between the healthy and NS group is significant, indicating that the disease has a distinct efect. This is expected because the disease introduces measurable changes that distinguish it from a healthy state. The comparison between the NS and CGP groups showed significant diferences, indicating that CGP treatment improved the demyelination in the spinal cord. Asterisks (*) denote statistically significant diferences ($p < 0.05$) between the experimental groups and are expressed as mean ± standard deviation (SD).

Table 4. The one-way ANOVA table for the quantitative analysis of EdU cell count which represents newly proliferated cells for to 18 and 35 days of induction.

Group comparison	Mean diference (18 days)	Mean diference (35 days)
Healthy vs. NS	5.67	3.0
Healthy vs. CGP	–32.0*	–41.67*
Healthy vs. Bac	1.0	–1.0
Healthy vs. CGP + Bac	–9.67	–12.33
NS vs. CGP	–37.67*	44.67 *
NS vs. Bac	–4.67	–4.0
NS vs. CGP + Bac	–15.33	–15.33
CGP vs. Bac	33.0	40.67*
CGP vs. CGP + Bac	22.23	29.33
Bac vs. CGP + Bac	–10.67	–11.33
F and*p* values	$F_{4,10} = 5.798$ $p < 0.05$	$F_{4,10} = 8.101$ $p < 0.05$

one-way ANOVA on EdU cell count on Day 18 showed a statistically significant diference ($F_{4,10} = 5.798, p = 0.0112$) between the groups (**Figure 7**). A one-way ANOVA revealed a significant efect of group on the value $F_{4,10} = 5.798$ for 18 days; $F_{4,10} = 8.101$, with $p < 0.005$. Post-hoc comparisons were performed using Tukey's test to correct for multiple comparisons. The analysis showed that the CGP group had significantly lower values compared to the Healthy group for both 18 (mean diference = –32.0, $p < 0.05$) and 35 Days (mean diference = –44.67, $p < 0.05$). No other comparisons reached statistical significance after correction (**Table 4**).

Mean diferences in EdU cell count were compared between groups using one-way ANOVA. Significant diferences were observed in most of the CGP groups; Healthy vs. CGP andNS vs. CGPin both 18 and 35 days and CGP vs Bac group in 35 days (*$p < 0.0001$ was considered significant). *$p < 0.05$ was considered significant. Abbreviations: NS = Normal Saline; Bac = Baclofen; CGP = CGP-55845.

The assessment of proliferating cells serves as an indicator of newly generated cells detected by Olig2 proliferation following treatment. This provides a basis for evaluating oligodendrocyte generation and its potential for remyelination. For Day 18 induction, it was noted that the Olig2 cell count in the CGP group had significantly increased ($p < 0.05$) compared to the other groups. For Day 35 induction, it was noted that the Olig2 cell count in the CGP group has significantly increased ($p < 0.05$) compared to the other groups (**Figure 8**).

A one-way ANOVA was conducted to examine the efect of diferent treatment groups on Olig2 cell count for 18 and 35 days. The result showed a highly significant efect of group on Olig2 cell count at both time points (18 days: $F_{4,10} = 518.8$) and 35 days ($F_{4,10} = 199.6$), $p < 0.05$, indicating substantial diferences among the groups (**Table 5**).

To further evaluate cellular proliferation in the spinal cord following induction, immunofluorescence staining was performed using EdU and Olig2 markers. **Figure 9** illustrates the representative histological images of spinal cord sections from all experimental groups for the 18-day group, and **Figure 10** illustrates the representative histological images of spinal cord sections from all experimental groups for the 35-day group.

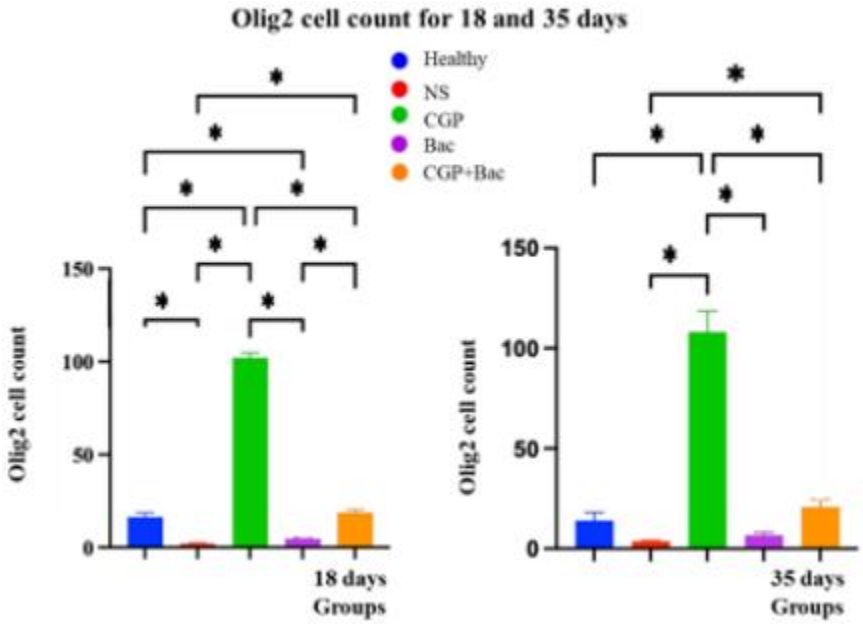

Figure 8. Olig2 cell count between 18 and 35 days for all experimental groups. Olig2 cell counts indicate the proliferative new generation of oligodendrocyte cells. The CGP group exhibited a significantly increased Olig2 cell count compared to other groups on both the 18th and 35th days, reflecting its therapeutic impact. The comparison between the healthy and NS group is significant, indicating that the disease has a distinct efect. This is expected because the disease introduces measurable changes that distinguish it from a healthy state. The comparison between healthy and CGP showed a significant diference, indicating that CGP might be efective in mitigating the disease progression. The comparison between the NS and CGP groups showed significant diferences, indicating that CGP treatment improved the demyelination in the spinal cord. Asterisks (*) denote statistically significant diferences ($p < 0.05$) between the experimental groups and are expressed as mean ± standard deviation (SD).

Table 5. The one-way ANOVA table for the quantitative analysis of Olig2 cell count for 18 and 35 days of induction.

Group comparison	Mean diference (18 days)	Mean diference (35 days)
Healthy vs. NS	14.33 *	10.33
Healthy vs. CGP	85.67 *	–94.0 *
Healthy vs. Bac	11.67*	7.33
Healthy vs. CGP + Bac	–2.33	–6.67
NS vs. CGP	–100.0*	–104.3 *
NS vs. Bac	–2.67	–3.0
NS vs. CGP + Bac	–16.67*	–17.0 *
CGP vs. Bac	97.33*	101.3 *
CGP vs. CGP + Bac	83.33*	87.33 *
Bac vs. CGP + Bac	–14.0*	–14.0
F and *p* values	$F_{4,10} = 518.8$ $p < 0.0001$	$F_{4,10} = 199.6$ $p < 0.0001$

Mean diferences in Olig2 cell count were compared between groups using one-way ANOVA. Significant diferences were observed in most of the CGP groups: Healthy vs. CGP and NS vs. CGP in both 18 and 35 days and CGP vs. Bac group in 35 days (*$p < 0.0001$ was considered significant). *$p < 0.05$ was considered significant. Abbreviations: NS = Normal Saline; Bac = Baclofen; CGP = CGP-55845.

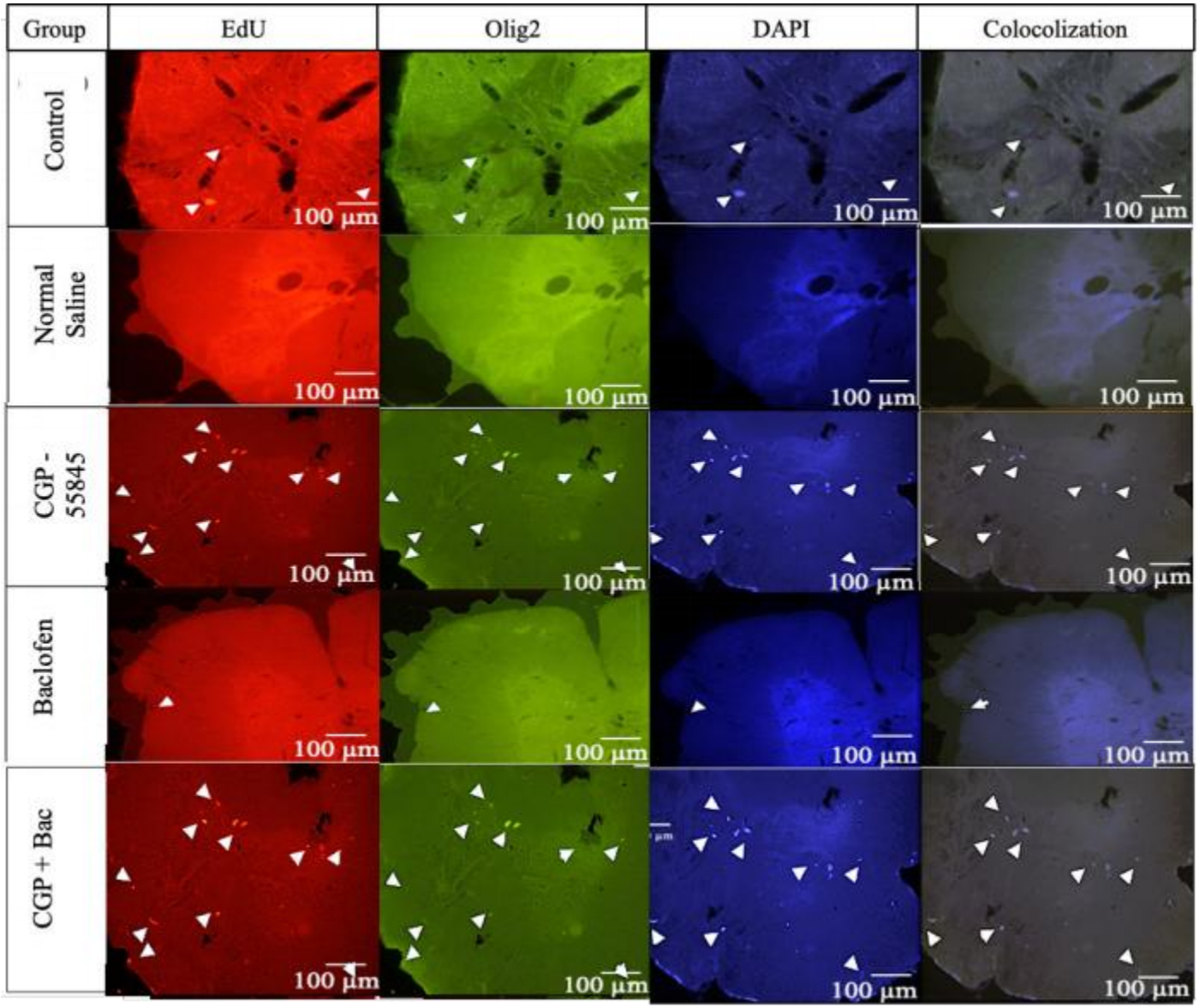

Figure 9. Immunofluorescence immunostaining of EdU and Olig2 cell count of the spinal cord section for 18 days. The histology sections ofall the groups were subjected to EdU, Olig2, and DAPI staining at 10× magnification. The Healthy group demonstrated no significant cell proliferation. Meanwhile, higher cell proliferation occurred in the CGP–55845 (CGP) group. The reduced cell proliferation was observed in the normal saline (NS) group, mild and moderate in the baclofen (Bac) group, and the CGP-55845 + baclofen (CGP + Bac) group, respectively. Note that EdU is to detect newly proliferated cells, and the overlapped EdU with Olig2 cells confirmed that oligodendrocytes can proliferate with the treatments. DAPI is a confirmatory detection of the nuclei. The arrows indicate the proliferation of cells in the lumbar section of the spinal cord.

The CGP group also showed significantly lower counts than the Bac and CGP + Bac groups. Similarly, the NS group had significantly lower Olig2 cell counts compared to Bac and CGP + Bac. For Day 35, similar trends were observed, with the CGP group having significantly lower Olig2 cell counts compared to Healthy, NS, Bac, and CGP + Bac groups (all $p < 0.05$). The findings indicate that CGP treatment leads to a significant reduction in Olig2 cell count, suggesting a potential inhibitory efect on Olig2-positive cells.

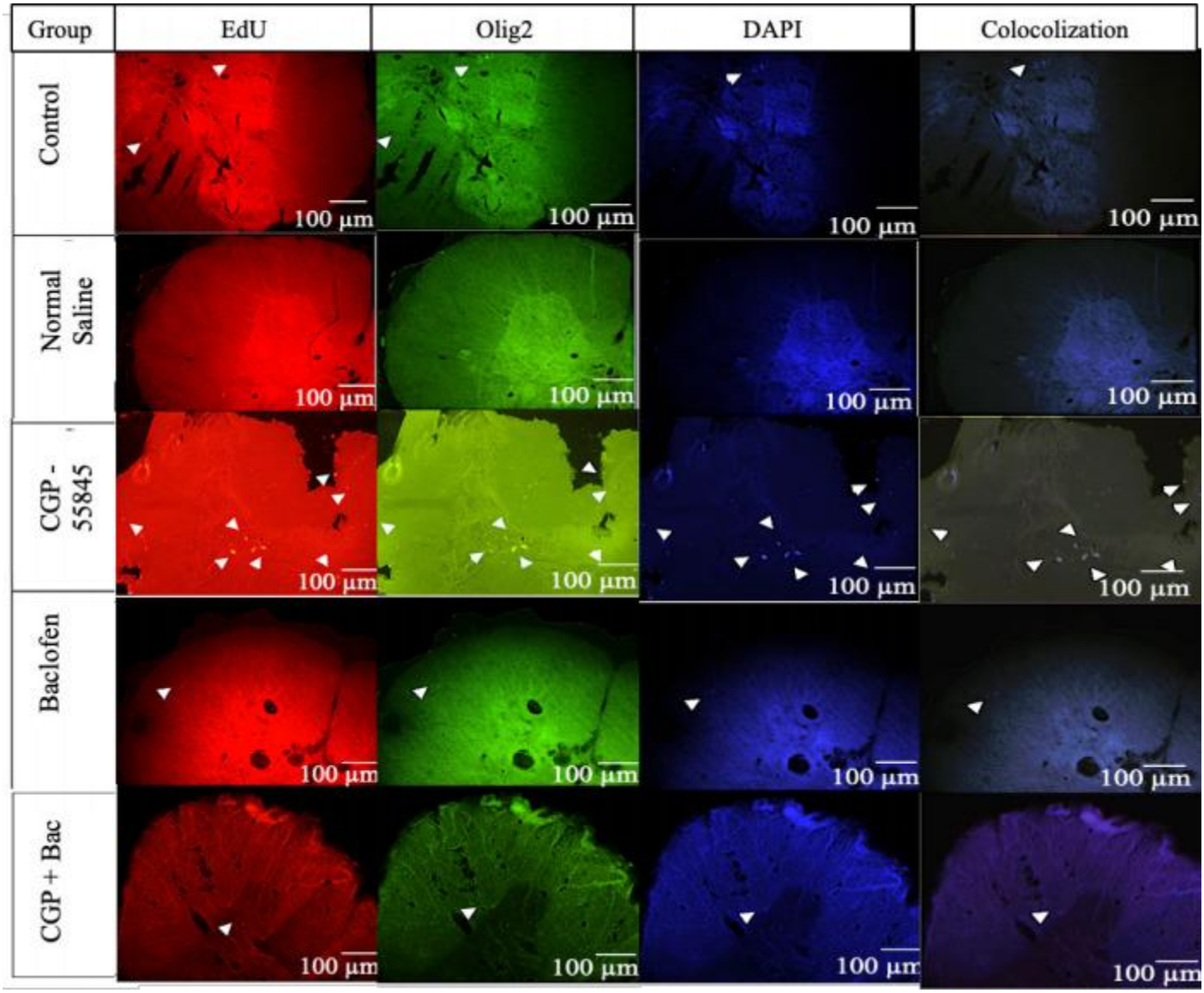

Figure 10. Immunofluorescence immunostaining of EdU and Olig2 cell count of the spinal cord section for 35 days. b) The histology sections ofall the groups were subjected to EdU, Olig2, and DAPI staining at 10× magnification. The Healthy group demonstrated no significant cell proliferation. Meanwhile, higher cell proliferation occurred in the CGP–55845 (CGP) group. The reduced cell proliferation was observed in the normal saline (NS) group, mild and moderate in the baclofen (Bac) group, and the CGP-55845 + baclofen (CGP + Bac) group, respectively. Note that EdU is to detect newly proliferated cells, and the overlapped EdU with Olig2 cells confirmed that oligodendrocytes can proliferate with the treatments. DAPI is a confirmatory detection of the nuclei. The arrows indicate the proliferation of cells in the lumbar section of the spinal cord.

Minimal cell proliferation was observed in the Healthy group, whereas the CGP group exhibited the highest cell proliferation. In contrast, the NS group showed reduced cell proliferation, while mild to moderate proliferation was observed in the Bac and CGP + Bac groups, respectively. The colocalization confirms that oligodendrocytes positively showed proliferation following induction and thus influence the regenerative capacity of oligodendrocytes.

3.5. $GABA_{BR1}$ protein expression in spinal cord

Western blot analysis was performed to assess the expression levels of the $GABA_{BR1}$ subunit protein in the spinal cord for each group. ß-actin was used as the loading control. For the Day 18 group, the $GABA_{BR1}$ protein expression was significantly increased in the CGP-treated group compared to the NS group. For Day 35 groups, $GABA_{BR1}$ expression remained elevated in the CGP group and was also significantly increased in the CGP + Baclofen group compared to both the NS group and healthy group ($p < 0.05$). Interestingly, the Baclofen only group showed intermediate levels of expression, but the increase was not statistically significant compared to controls. The CGP group showed the highest mean relative protein level, suggesting that CGP treatment upregulated $GABA_{BR1}$ expression. No significant diferences were observed between the other treatment groups (Baclofen alone or CGP + Baclofen) and the healthy group. The mean relative $GABA_{BR1}$ subunit protein level was used to detect the expression of $GABA_{BR1}$ in the lumbar section of the spinal cord. Remyelination is potentiated by the expression of $GABA_{BR1}$ subunit protein level (**Figure 11**).

The mean relative $GABA_{BR1}$ subunit protein level was used to assess expression in the lumbar spinal cord. The CGP group showed increased $GABA_{BR1}$ levels compared to the Healthy and NS groups. Increased $GABA_{BR1}$ subunit protein levels in the CGP group suggest that this receptor may play a facilitative role in remyelination. This could point to $GABA_{BR1}$ as a therapeutic target. No significant diferences were observed between the other treatment groups (Baclofen alone or CGP + Baclofen) and the healthy control. For Day 35 groups, $GABA_{BR1}$ expression remained elevated in the CGP group and was also significantly increased in the CGP + Baclofen group, compared to both NS and healthy groups. Interestingly, the Baclofen only group showed intermediate levels of expression, but the increase was not statistically significant compared to controls.

The results of the one-way ANOVA on the mean relative $GABA_{BR1}$ subunit protein level on Day 18 showed that there was a statistically significant diference ($F_{4,10} = 4.428$, $p = 0.0257$) between the groups at individual time points (**Table 6**). Post-hoc Turkey's test revealed that the mean relative $GABA_{BR1}$ subunit protein level significantly increased after the treatments in the NS group relative to that of the CGP group ($p < 0.05$, 95% CI –2.433 to –0.2341). There was no significant diference detected in all other group comparisons.

The results of the one-way ANOVA on the mean relative $GABA_{BR1}$ subunit protein level on Day 35 remained statistically significant diference ($F_{4,10} = 5.834$, $p = 0.0109$) between the groups (**Table 7** and **Figure 11**). Post-hoc Turkey's test

Table 6. The one-way ANOVA table for the quantitative analysis of mean relative $GABA_{BR1}$ subunit protein level, which represents $GABA_{BR1}$ subunit protein level expression from day 1 to day 18 ($n = 15$).

	Sum of squares	df	Mean square	*F*	Sig.
Between groups	2.964	4	0.7410	$F_{4,10} = 4.428$	$p = 0.0257$
Within groups	1.673	10	0.1673		
Total	4.637	14			

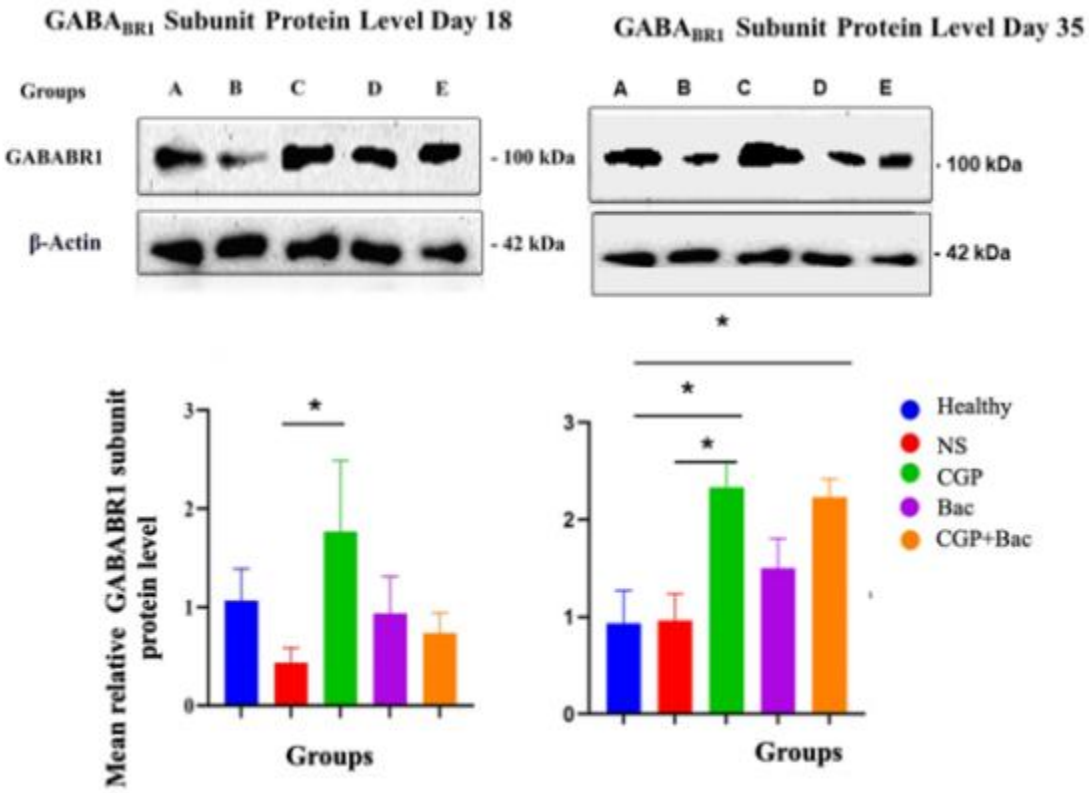

Figure 11. GABA$_{BR1}$ subunit protein level between 18 and 35 days for all experimental groups in western blot analysis. GABA$_B$ receptors are expressed on neural progenitor cells to influence the proliferation and diferentiation of neural stem cells into oligodendrocyte progenitor cells (OPCs), which are crucial for remyelination. The CGP group exhibited a significantly increased GABA$_{BR1}$ subunit protein level compared to the NS group on both the 18th and 35th days, reflecting its therapeutic impact. The comparison between healthy and CGP showed a significant diference, indicating that CGP might be efective in mitigating the disease progression. The comparison between the NS and CGP groups showed significant diferences, indicating that CGP treatment improved the demyelination in the spinal cord. Asterisks (*) denote statistically significant diferences ($p < 0.05$) between the experimental groups and are expressed as mean ± standard deviation (SD).

revealed that the mean relative GABA$_{BR1}$ subunit protein level was significantly increased after the treatments in the following groups comparisons: Healthy vs. CGP (p = 0.0324, 95% CI –2.691 to –0.1094); Healthy vs. CGP + Bac (p = 0.482, 95% CI –2.591 to –0.0094); and NS vs. CGP (p = 0.0370, 95% CI –2.657 to –0.0761). There was no significant diference detected in any other pairwise group comparisons.

These findings suggest that CGP treatment significantly reduced GABA$_{BR1}$ expression relative to both healthy and NS groups by Day 35, while CGP + Bac treatment also showed a significant result.

Table 7. The one-way ANOVA table for the quantitative analysis of mean relative GABA$_{BR1}$ subunit protein level, which represents GABA$_{BR1}$ subunit protein level expression from day 1 to day 34 (n = 15).

	Sum of squares	df	Mean square	F	Sig.
Between groups	5.383	4	1.346	$F_{4,10}$ = 5.834	p = 0.0109
Within groups	2.307	10	0.2307		
Total	7.689	14			

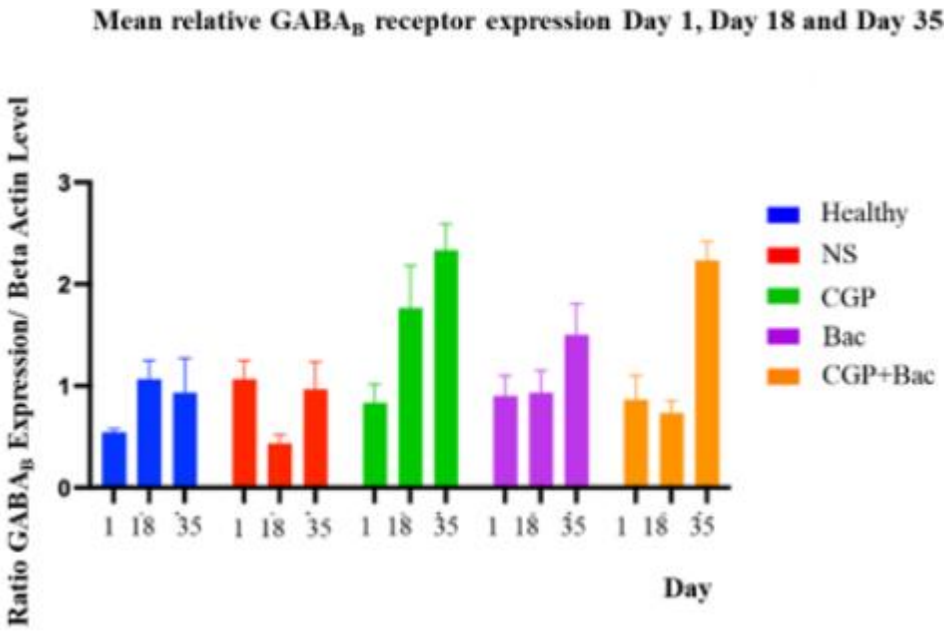

Figure 12. Mean relative GABA$_B$ receptor expression between Day 1, Day 18, and Day 35 in the groups (n = 30). The CGP group showed a gradual increase in GABA$_B$ receptor expression over time, and the CGP + baclofen group exhibited a lower expression on Day 18 compared to the CGP-only group but then increased again by Day 35. Other groups did not demonstrate marked changes or a consistent increasing trend over the 35 days. Although an upward trend in GABA$_B$ receptor expression was observed, especially in the CGP-treated groups, the diferences were not statistically significant. The results are expressed as mean ± standard deviation (SD).

To assess changes in each group, a mixed ANOVA was conducted and analysis reported no significant efect of the GABA$_B$ receptor expression (row) [F (1.184, 2.369) = 9.056, ε = 0.5922, p > 0.05], Time (column) [F (1.147, 2.294) = 10.23, ε = 0.2867, p > 0.05], or row x column interaction [F(1.294, 2.588) = 6.465, ε = 0.1618, p > 0.05]. Despite the absence of significant overall interaction, pairwise comparison analysis showed an increasing trend in GABA$_B$ receptor expressions between Day 35 and Day 18 in the treatment groups, which may have biological relevance, as shown in **Figure 12**.

CGP shows the most pronounced increase in GABA$_B$ receptor expression at Day 18 and Day 35 compared to all other groups. Baclofen shows a moderate increase or stable trend, but not as marked as CGP, as baclofen likely activates the receptor without increasing its expression. CGP + Bac group shows sustained elevation, particularly at Day 35, though not as high as CGP alone on Day 18. The results on receptor expression at both Day 18 and Day 35 compared to other groups suggest a potential modulatory efect by CGP and Baclofen. A similar upward trend was observed in the CGP + Bac group, especially at Day 35, indicating possible additive or synergistic interaction between treatments. The result supports the hypothesis that CGP modulates receptor availability.

4. Discussion

4.1. Role of $GABA_B$ receptor modulation

$GABA_B$ receptors are essential for neural processes, such as regulating neuronal excitability and synaptic plasticity, mainly through inhibitory neurotransmission [30]. Activation of these receptors decreases neuronal excitability, potentially influencing myelination by modulating OPC activity [31]. The statistically significant increase in EAE scores for all experimental groups in the present study confirms the success of EAE induction. This means that the disease model efectively mimicked MS-like symptoms. EAE caused immune cells to breach the blood–brain barrier, leading to demyelination through a series of inflammatory events that damaged myelin sheaths, creating lesions typical of MS. We investigated the efects of both $GABA_B$ receptor agonists and antagonists to determine whether activation or inhibition of the $GABA_B$ receptor stimulated the proliferation of oligodendrocytes and promoted their maturation into myelinating oligodendrocytes, thereby promoting remyelination. Previous studies have shown that the $GABA_B$ receptor agonist, baclofen, accelerated OPC diferentiation and remyelination in purified rat OPC cultures [16,22]. However, our findings suggest that the $GABA_B$ receptor antagonist, CGP-55845, more efectively promoted remyelination and functional recovery. This suggests that $GABA_B$ receptor inhibition, rather than activation, better facilitates remyelination within the complex inflammatory condition of EAE.

4.2. EAE progression and inflammation induced demyelination

In the established mouse model, the course and symptoms of EAE followed a typical pattern as documented by previous studies [27,32]. EAE was originally reported more than 90 years ago [33,34] and the model showed a classical EAE with clinical symptoms manifesting after MOG(35–55) peptide induction. EAE provides a valuable framework for understanding the intricate immunopathogenic mechanisms. In this model, immune cells cross the blood–brain barrier, triggering inflammatory responses and causing damage to the myelin sheath [27]. One such mechanism involves perforin, a pore-forming protein released by cytotoxic lymphocytes, which can induce lysis of oligodendrocytes. By forming pores in the oligodendrocyte membrane, perforin disrupts cellular integrity, leading to cell swelling, membrane disruption and eventual cell death, confirming the relevance of this model to MS pathology [35,36]. Activated T cells, along with other immune cells, infiltrate the CNS due to a compromised blood–brain barrier, which is normally protective. These T cells recognize and interact with specific myelin proteins such as MBP, proteolipid protein and myelin oligodendrocyte glycoprotein. This interaction triggers an immune response against these myelin components, leading to the formation of immune complexes and the involvement of B cells and macrophages. This process results in the release of pro-inflammatory cytokines and chemokines. Macrophages and phagocytes contribute to the demyelination process. The inflammatory cascade and immune cell activity lead to the destruction of myelin sheaths and nerve fibers

resulting in the formation of characteristic plaques or lesions, or results comparable to the results of MOG induction, which also result in measurable locomotor impairments [27].

EAE can be induced either by the adoptive transfer of CD4 + T cells specific to myelin epitopes or through sensitization with myelin proteins. Chemokines and proinflammatory cytokines released during EAE contribute to CNS damage and myelin degradation. The hallmark of EAE is multifocal demyelination in the brain and spinal cord, driven by an immune response targeting myelin proteins. As a well-established experimental model of MS, EAE has been extensively utilized in numerous studies [27,37–40].

Despite the generally limited extent of remyelination in the EAE model, several studies have demonstrated the proliferation of OPCs and their diferentiation into myelin-producing oligodendrocytes within the damaged CNS of MOG-induced EAE rats, ofering insights into potential mechanisms of repair [41]. In the present study, we hypothesized that oligodendrocyte-mediated remyelination, which initially forms a thin layer of myelin around the axon, is linked to a distinct process of myelin loss. This suggests that demyelination may difer significantly from remyelination, with oligodendrocytes possibly retracting myelin sheaths in a controlled manner during demyelination, producing the characteristic thin myelin layer observed during remyelination [29].

CGP-55845 treatment induces measurable improvements in neurobehavioral performance. One of the criteria for evaluating disease progression in the EAE model is a decrease in body weight. This change is thought to result from low-grade inflammation, a known contributor to neurodegenerative processes [41]. Excessive weight gain triggers the activation of proinflammatory cytokines and microglia, leading to an increase in the expression of TNF-α [42]. That study also highlights the fact that weight gain in animals is sometimes associated with the activation of microglia, which in turn elevates TNF-α expression [43]. TNF-α plays a critical role in neurodegeneration, as its expression in the CNS stimulates the sympathetic nervous system, promotes oxidative stress, and contributes to neuronal death [42]. Additionally, TNF-α production in the CNS leads to myelin vacuolation and oligodendrocyte death [43]. We propose that TNF-α, produced by microglia, acts as a catalyst for increased blood–brain-barrier permeability, exacerbating the progression of EAE.

The rats in the CGP group, which showed lower daily body weight and weight gain, are consistent with findings from a study by Bizzozzero-Hiriart et al. [44]. In their study, neonatal male and female mice received saline or the $GABA_B$ antagonist CGP 55845 (1 mg/kg, s.c.) from postnatal day 2 (PND2) to PND6, 3X/day (at 0800, 1100, and 1800 h) [44]; the mice showed a weight reduction pattern that was similar to that ofthe rats in our study.

Weight loss can begin 1–2 days before the onset of EAE symptoms, typically occurring between days 6 and 14. In the present study, rats treated with MOG (35–55) exhibited a substantial increase in EAE scores, indicating the progression of clinical symptoms. However, unlike previous findings by Zorzella-Pezavento et

al., in which EAE progression was associated with weight loss, our study showed an accompanying weight gain during disease progression [45]. This discrepancy suggests that weight changes in EAE may vary depending on specific experimental conditions and interventions.

4.3. Implications of $GABA_B$ antagonism

It is interesting to note that the administration of the $GABA_B$ antagonist in our study positively afected neurobehavioral outcomes and reduced disease severity in the EAE model, suggesting that blocking $GABA_B$ receptors supports oligodendrocyte development and remyelination. The EAE-induced models showed an indirect indicator for neuroinflammation and neurodegeneration, in the sense that weight loss is consistent with the disease progression and disturbances. The treated groups exhibited less weight gain than the healthy groups, and the gradual restoration suggests that the interventions supported the recovery process by alleviating neuroinflammation. The observed weight gain in our study could be attributed to the neuroprotective efects of the $GABA_B$ antagonist, which may mitigate the typical weight loss associated with EAE progression. Notably, the Day-18 treatment course may have coincided with the peak of the disease progression. These findings highlight the potential therapeutic benefits of targeting $GABA_B$ receptors in MS, not only for promoting remyelination but also for improving overall disease outcomes. Further research is needed to elucidate the mechanisms by which $GABA_B$ receptor antagonism influences weight changes and neuroinflammation in EAE.

The ratsin ourNS group showed increased EAE symptoms and were significantly diferent from those in the Healthy group. This suggests that the changes in EAE symptoms were influenced by the active development of the illness, suggesting that EAE induction reliably elevated the daily scores relative to those of the Healthy group. Although the statistics provided did not show significant diferences between the NS group and the drug-treated groups in terms of overall neurobehavioral outcomes and disease severity, the static scoring in the NS group (persistent from day 11 postinduction) highlights a lack of recovery or progression in this group. This indicates that without intervention, disease severity remains constant. In contrast, the EAE symptom scores decreased in CGP, CGP + Bac, and Bac groups after treatments, suggesting that the clinical progression of the disease had improved. The activation of $GABA_B$ receptor antagonists afected early processes, some of which occurred before synapse formation. These complex processes associated with oligodendrocyte development include proliferation, migration, diferentiation, and myelin production [46].

Based on a study by Rossi et al. (2012), reduced GABA-mediated neuronal inhibition, especially in the grey matter of the brain in MS, suggested that GABA signaling is important in the clinical course of EAE in mice. The reduced inhibition suggests that GABAergic dysfunction serves as a biomarker for progression in EAE. Therefore, enhancing the GABA signaling could mitigate the severity of EAE, potentially delaying or altering the clinical course. In MS, late neurodegeneration and a progressive clinical course are correlated with the frequency of inflammatory events

during the early stages ofthe illness. During acute MS attacks, inflammatory cytokines can increase glutamate-mediated synaptic transmission while decreasing GABA-mediated synaptic signaling. This can lead to imbalanced synaptic hyperexcitation and potentially excitotoxic neurodegeneration [47]. These processes accelerate the advancement of EAE illness.

Additionally, our findings suggest that $GABA_B$ receptor antagonists may play a protective role by supporting oligodendrocyte development and remyelination. This could mitigate some of the neurodegenerative efects observed in MS, providing a potential therapeutic avenue for managing the disease.

The presence of demyelinating plaque in the spinal cord indicates the development of EAE in a rat model that closely mimics MS in humans [48]. Our findings demonstrated that CGP-55845 treatment significantly improved spinal cord histology and alleviated or reversed EAE symptoms in the treated rats. Western blot analysis further supported these results, showing increased counts of EdU and Olig2 cells and elevated expression levels of the $GABA_{BR1}$ subunit protein after CGP-55845 treatment. This suggests that remyelination in the spinal cord is potentiated by GABA antagonists and the activation of the $GABA_{BR1}$ receptor [49]. Additionally, Wang et al. 2023 reported that administration of LPC to Tregs-depleted mice induced larger lesions, shown by LFB staining, than in the PBS-treated group. Comparing the LPC group to the sham group, Western blot analysis revealed reduced MBP expression, which was consistent with the results of immunofluorescence and LFB labeling. Treg depletion decreased the protein expression of MBP more than PBS-LPC treatment. After LPC injection, there was also a reduction in the expression of axonal injury markers. The reduction produced by Tregs led to even lower expressions of axonal injury markers than did PBS-LPC treatment [49].

These findings underscore the potential ofCGP-55845 in promoting remyelination and improving clinical outcomes in EAE, and highlight its therapeutic promise for MS. Further research is warranted to elucidate the precise mechanisms by which $GABA_B$ receptor antagonism influences neuroinflammation and remyelination.

Exogenous GABAinfluences myelination by increasing MBPlevels and the myelin sheath segments around axons. These efects are likely direct, as GABA stimulation of cultured OPCs accelerates their diferentiation into mature oligodendrocytes (OLs) by raising MBP levels in the cell periphery, suggesting local translation of MBP in these regions [22]. To assess the impact of treatments on myelination, spinal cord tissue slices were stained with LFB. Although LFB stain intensity was lower in EAE mice, it significantly increased with CGP-55845 administration, showing greater intensity in CGP-treated mice than did other treatments. The results of another study that used an EAE rat model showed that those treated with NSCs showed higher staining intensity than did those receiving other treatments, indicating that endogenous remyelination was triggered by those interventions [50].

CGP-55845 treatment also yielded upregulation of $GABA_{BR1}$ protein levels. These findings suggest that GABAB receptor antagonism modulates not only OPC proliferation but also their diferentiation and integration into remyelination pathways. Furthermore, the overlap of EdU+ and Olig2+ cells indicated active oligodendrocyte diferentiation in treated animals. Other studies using qRT-PCR

examined myelin-associated gene expression, such as MBP and myelin MOG, in spinal cord and brain tissue, which showed the expressions of Erk2, Krox-20, MBP, and MOG were significantly upregulated in NSC-treated tissue [50]. This suggests that increased LFB staining intensity correlates positively with upregulated myelin-associated gene expression, leading to remyelination. Consistent with these findings, GABA antagonists act as positive modulators of myelination through $GABA_{BRs}$, thereby improving EAE symptoms.

EdU staining has been used to detect newly proliferated cells, and the overlap of EdU with Olig2 confirms that oligodendrocytes proliferated with the treatments [28]. The survival of oligodendrocytes and the capacity of OPCs to produce new myelinating cells can be attributed to remyelination from the treatments. The primary mechanism against demyelination is the remyelination of mature oligodendrocytes [51]. Oligodendrocytes express both monoamine oxidase B and glutamate decarboxylase. Further evidence of GABA synthesis by oligodendrocytes, presumably for appropriate diferentiation and maturation, comes from the detection of GABA in OPCs, NG2+ cells, and OLs at various maturity stages. Thus, GABA production by Bergmann glia and Schwann cells' response to this neurotransmitter suggests an autocrine/paracrine signaling system that plays a role in axon-glia communication [52]. This study postulates that GABA antagonists modulate oligodendrocyte and glial cell proliferation, diferentiation, and myelination. Blocking $GABA_{BRs}$ afects the number of oligodendrocytes in slices and positively regulates myelination in situ in spinal cord slices [52].

GABA indirectly influences muscarinic receptors by regulating acetylcholine release, afecting downstream signaling pathways, including muscarinic receptor activation in OPCs and oligodendrocytes [48,53]. The G-protein regulator receptors of the striatum modify GABA transmission, and the endocannabinoid *N*-palmitoylethanolamine activates those receptors, increasing GABAergic synaptic activity [54].

Several neurotrophic-factor signaling pathways, including IGF-1 and brain-derived neurotrophic factor (BDNF), induce OPC maturation or boost myelin protein production and lipid synthesis, promoting remyelination [35,36]. GABA promotes growth in zebrafish larvae by inducing IGF-1 expression via $GABA_A$ and $GABA_B$ receptors [21,55]. We postulate that GABA is also involved in remyelination through IGF expression, myelin staining, MBP and proteolipid protein expression, and the number of oligodendrocytes and their precursors [56]. BDNF, crucial for GABAergic synaptic transmission, is released in an activity-dependent manner. Although proBDNF binds to the p75 neurotrophin receptor, mature BDNF functions through its receptor TrkB. Those signaling pathways regulate GABA-related neurotransmission under physiological conditions [57,58]. BDNF is essential for CNS myelination and is expressed in neurons, glial cells, and myelin-forming oligodendrocytes [59].

During development, OPCs express GABA receptors that are highly sensitive to ambient GABA levels. GABA antagonists might disrupt this signaling in a way that favors proliferation over diferentiation, which may occur due to preferences over the timing of development and to the sensitivity of the receptor subtype. Oligodendrocytes and OPCs are responsible for myelin production; oligodendrocytes

are damaged in MS, leading to demyelination. Cannabinoids also have been shown to promote the diferentiation of OPCs into mature oligodendrocytes, enhancing remyelination processes and the postulated receptor mechanism through cannabinoid receptors [60,61]. Cannabinoid-receptor activation is known to support remyelination by promoting OPC diferentiation and protecting oligodendrocytes from inflammatory damage by counteracting oxidative stress. The receptor can activate the interneurons and may afect GABA release, which could synergize with $GABA_B$ receptor antagonism in promoting OPC proliferation [60,61,64]. Our results showed significant remyelination, with higher LFB intensity in the CGP-treated group than in other groups, particularly in the spinal cord. $GABA_B$ antagonists might work by expanding the OPC population through increased proliferation and by ensuring an adequate supply of precursors for myelin repair. The higher level of myelin expression in our CGP-treated rats suggests that the cells promote remyelination and myelin oligodendrocyte diferentiation. Histological analysis revealed that CGP-55845 significantly improved spinal cord structure, reversing demyelination in EAE-induced rats. $GABA_B$ antagonism alters the microenvironment, potentially increasing the oligodendrocytes. Increasing oligodendrocyte diferentiation provides new mature oligodendrocytes that can replace those lost by T cell cytotoxic mechanisms including perforin-mediated lysis and granzyme-mediated apoptosis [35,36]. Western blot analysis showed high expression levels of the $GABA_{BR1}$ subunit protein in CGP-treated rats. Altogether, our results suggest that CGP-55845 plays a crucial role in promoting proliferation and remyelination.

5. Conclusions

The results of the treatment with the $GABA_B$ receptor antagonist, CGP-55845, show significant potential in alleviating the pathology of EAE and reversing disease progression in the CNS. By promoting oligodendrocyte diferentiation and maturation into myelinating cells, $GABA_B$ receptor antagonists may act through specific $GABA_{BR1}$ receptors. This provides preliminary evidence supporting the use of $GABA_B$ receptor antagonists as treatments for MS.

When comparing the disease progression at 18 and 35 days, two phases are seen. On day 18, the disease progression reaches its maximum severity, and by day 35, the disease is in a chronic phase, indicating a shift in the pathological process.

These findings suggest that $GABA_B$ receptor antagonists could be viable therapeutic candidates for MS. Our results suggest that selective $GABA_B$ receptor antagonist CGP-55845 plays a significant role in promoting neural stem cell proliferation, the oligodendrocyte precursor cells, and remyelination in the EAE model of MS. Further research is needed to clarify the cellular and molecular mechanisms, as well as the related signaling pathways, to optimize myelin regeneration. Understanding these processes will be crucial for developing efective treatments that leverage $GABA_B$ receptor antagonism to promote remyelination and improve clinical outcomes in MS patients.

However, several limitations should be considered. The study was conducted in a rodent EAE model, which does not fully replicate the human MS pathology. The use of Olig2 as a single marker for oligodendrocyte diferentiation may limit

the interpretation of remyelination, incorporating additional lineage specific markers could provide a more robust characterization. Additionally, the possible efects of CGP-55845 on other CNS cell types were not assessed and could confound the interpretation. Environmental variables such as stress during EAE scoring may also cause bias. Future studies should explore a broader cellular marker, dose-response relationships and examine long term outcomes with other MS models to better translate findings into clinical applications.

Author contributions: Conceptualisation, NS and NAZ; methodology, NS and NG; software, NS and NAZ; validation, NS and NAZ; formal analysis, NAZ and NS; investigation, NS and NAZ; resources, NAZ; data curation, RZ, NS and NAZ; writing-original draft preparation, NAZ; writing-review and editing, NS; visualisation, NAZ; supervision, NS, NG and KM; project administration, NS; funding acquisition, NS.

Ethical approval: This research was funded by the Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme (FRGS), grant number FRGS/1/2020/SKK0/USM/02/29, administered through Universiti Sains Malaysia. The article processing charge (APC) was funded by the School of Medical Sciences, Universiti Sains Malaysia.

Informed consent statement: Informed consent was obtained from all subjects involved in the study.

Availability of data and materials: Data generated and analysed in this study are included within the published article, and no external public datasets were used.

Conflict of interest: The authors declare no conflict of interest.

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