Effects of Low Field Magnetic Stimulation on Brain Remyelination and Cognitive impairment in the Chronic Cuprizone Demyelination Mouse Model of Multiple Sclerosis

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By

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ABSTRACT

Multiple Sclerosis (MS), a chronic inflammatory demyelinating disease of the Central Nervous System (CNS), is recognized as the leading cause of disability in young adults in Canada. The pathological features of MS include neuroinflammation, demyelination, and oligodendrocyte (OL) loss. Cognitive impairment (CI) and depression are the most common neuropsychiatric symptoms and major determinants of MS disability. Despite the broad and severe spectrum of signs and symptoms, we are still missing effective treatment methods that can be applied to treat MS.

Low field magnetic stimulation (LFMS) is a novel non-invasive neuromodulation technology. A few clinical and animal studies have shown that LFMS has beneficial effects on emotional disturbances and cognitive function. Our research has shown that LFMS ameliorated cuprizone (CPZ)-induced working memory deficits and depression-like behaviours in the mice. The current study aimed to assess the effects of LFMS on cognition and remyelination in a CPZ-induced chronic demyelination model of MS.

Eight-week-old female C57BL/6 mice were fed with 0.2% of CPZ (w/w) for 12 weeks (12w) to induce chronic brain demyelination. The mice resumed the regular diet and received 20-min LFMS or Sham treatment every day for five days a week. The treatments lasted for two (14w) or four weeks (16w) to study the effects of LFMS on locomotor functions, anxiety and depression-like symptoms, as well as working memory using behavioural tests at the different time points (12w, 14w, and 16w). The animals were then euthanized, and the brain samples were collected and stored at -80°C for future experiments (Western blots and immunohistochemistry).

The results showed that chronic CPZ administration led to working memory deficits and depression-like behaviours. The gross locomotor function and anxiety-like behaviours were not affected by CPZ. LFMS treatment significantly enhanced the expression of myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). LFMS also increased the expression of Glutathione S-transferase (GST-π), a mature OL marker. LFMS reduced the level of Glial Fibrillary Acidic Protein (GFAP), an activated astrocyte marker, and pro-inflammatory factor Tumor necrosis factor-alpha (TNFα). There was a significant reduction in the number of overall OL lineage cells labelled with Olig-2 (Oligodendrocyte Transcription Factor 2). A significantly enhanced expression of TGF-β (Transforming Growth Factor beta) and the receptors (TGF-β R1 and TGF-β R2) involved was reported.

Our results show that LFMS enhanced cognitive function and alleviated depression-like behaviours. LFMS facilitated the remyelination process in mice with chronic demyelination. LFMS may exert its therapeutic effects by reducing neuroinflammation and promoting OL regeneration through the TGF-β pathways. These results suggest that LFMS can be a promising therapeutic method for depression and cognitive impairment in MS patients. In addition, LFMS may also facilitate remyelination through its neuroprotective and immunomodulating effects, but this remains to be shown. Further studies are warranted to understand the detailed molecular mechanisms which facilitate the remyelination processes and behavioural deficits.

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LIST OF ABBREVIATIONS

BSA: Bovine Serum Albumin

CI: Cognitive Impairment

CNS: Central Nervous System

CPZ: Cuprizone

CPZT: Cuprizone (LFMS) Treatment

DMD: Disease-Modifying Drugs

GFAP: Glial Fibrillary Acidic Protein

GST-π: Glutathione S-Transferase pi

LFMS: Low Field Magnetic Stimulation

MBP: Myelin Basic Protein

MOG: Myelin Oligodendrocyte Glycoprotein

MS: multiple sclerosis

OFT: Open Field Test

Olig-2: Oligodendrocyte Transcription Factor 2

PBS: Phosphate Buffered Saline

PFA: Paraformaldehyde

PFC: Prefrontal Cortex

PIC: Protease Inhibitor Cocktail

PPMS: Primary-progressive multiple sclerosis

RRMS: Relapsing-remitting multiple sclerosis

SPMS: Secondary-progressive multiple sclerosis

TGF-β1: Transforming Growth Factor beta 1

TGF-β-R1: Transforming Growth Factor beta receptor 1

TGF-β-R2: Transforming Growth Factor beta receptor 2

TNFα: Tumor Necrotizing Factor-alpha

TST: Tail Suspension Test

1 INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic neurological disorder that causes catastrophic damages to the central nervous system (CNS) and a broad spectrum of disabilities [1]. The core symptoms and signs of MS include muscle weakness and spasms, sensory and balance impairments, urinary incontinence, visual problems and blindness, fatigue and pain, as well as emotional and cognitive deficits [2]. The most common pathological changes of MS are neuroinflammation, demyelination, and neuronal and oligodendrocyte (OL) damages (**Figure 1.1**) [3].

Canada has one of the highest MS prevalence in the world, with 290 cases per 100,000 population [4]. There are more than 90,000 patients in 2018 and is increasing in recent years in Canada [4] [5]. It is estimated that about 50% of patients need help walking within 15 years after the onset of the disease [6]. A summary of the countries with the highest prevalence rates of multiple sclerosis are shown in the table below:

Table 1: Countries with the highest prevalence rates of Multiple Sclerosis.

Regions	Case per 100,000 population
Canada	291
San Marino	250
Denmark	227
Sweden	189
Hungary	176
Cyprus	175
United Kingdom	164
Czech Republic	160
Norway	160
Germany	149

Source: https://www.healthline.com/health/multiple-sclerosis/facts-statistics-infographic#3

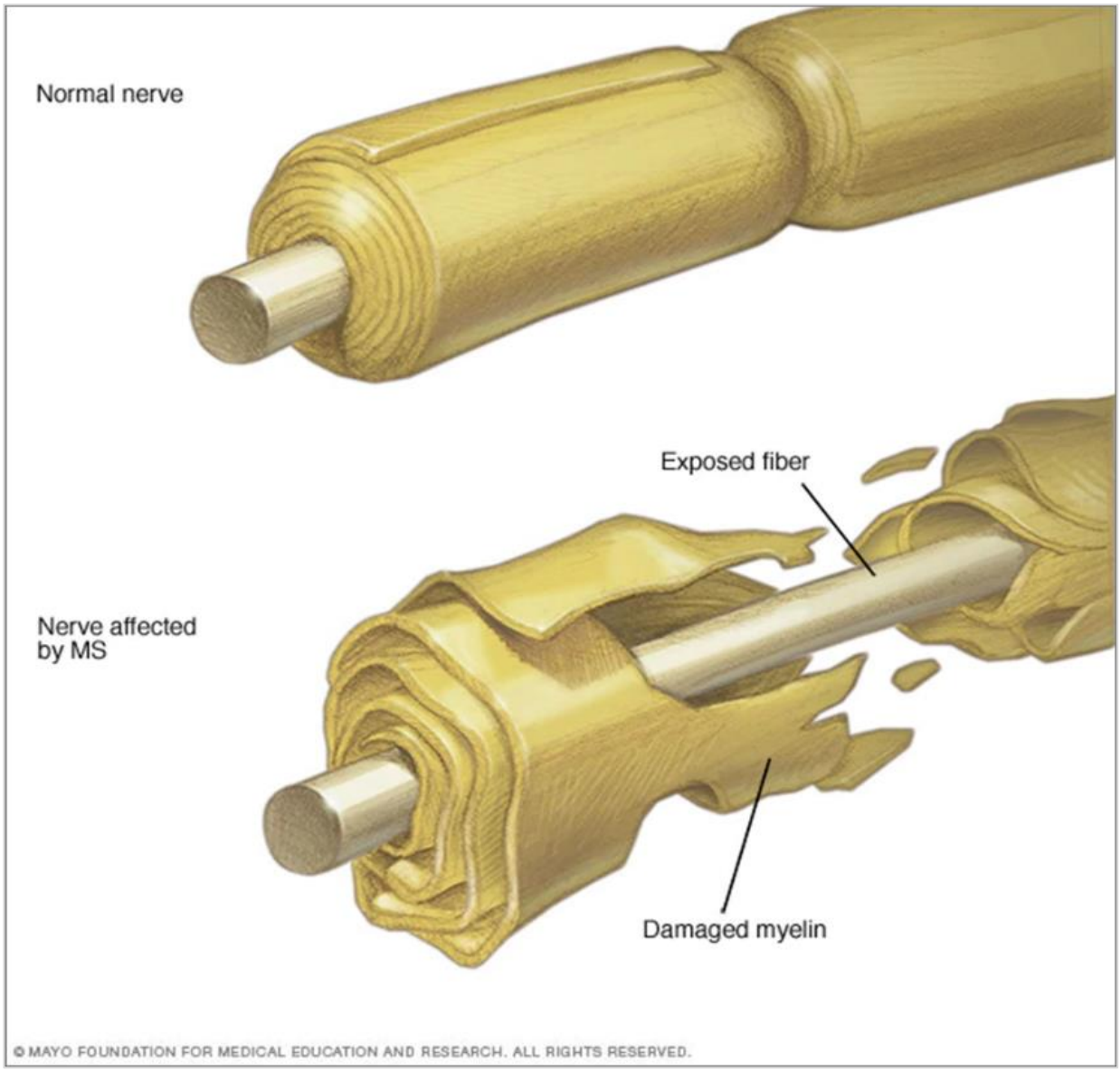

Figure 1.1: The normal and affected nerve in multiple sclerosis (MS). In multiple sclerosis, the protective coating myelin sheath (top) around axons is damaged (bottom) and may eventually be lost entirely. Unmyelinated axons are vulnerable to attacks and ultimately degenerate. [Ref: https://www.mayoclinic.org/diseases-conditions/multiple-sclerosis/multimedia/multiple-sclerosis/img-20006188]. Used with permission from the copy right owner MAYO CLINIC.

1.1.1 The clinical features of MS

Clinically, MS can be divided into asymptomatic, prodromal, and symptomatic phases [7, 8]. The relapsing symptoms of MS usually develop gradually and cause long-lasting neural damages in remitting stages called asymptomatic scarring of the nerve tissue [8]. The majority of patients (~ 85%) start with relapsing-remitting MS (RRMS), in which remissions follow the recurring symptoms (Figure 1.2A) [9]. The rest of the patients (~ 15%) have a progressive decline from the onset of the disease without remissions, resulting in a diagnosis of primary progressive MS (PPMS) (Figure 1.2B [9]). Up to 50% of patients with RRMS develop secondary progressive MS (SPMS) within 10-15 years when their symptoms persist without full remission [9, 10] (Figure 1.2C).

1.1.1.1 Relapsing-remitting MS

The overall course of MS is thus classified as 'relapsing-remitting' when the disease exhibits only relapses and remissions [11, 12]. Most often, RRMS starts with repeated neurologic episodes followed by partial or complete remission without new symptoms [13]. In RRMS, the typical lesion is inflammatory demyelination in the white matter (WM) of the CNS featured as diminishing myelin sheath around preserved axons [14]. Relapses are due to demyelination, followed by activated T cells entering the WM of CNS from blood [15-17]. Remission occurs when the immune attack is subsided, and remyelination is initiated by oligodendrocyte progenitor cells (OPC) [18, 19].

1.1.1.2 Primary-progressive MS

Progressive MS (PMS), including primary progressive MS (PPMS) and secondary progressive MS (SPMS), starts with or without relapses and remissions stage, followed by a progressive stage without remission. In PPMS, there is a "skipping" of the usual relapsing-remitting phase [13] in

which patients show a slow and steady functional decline from the time of its onset. Symptoms and severity of disability continue worsening with no or short remission. The neuropathology of PPMS is characterized by obvious GM demyelination in the cerebral and cerebellar cortex [20, 21]. The progressive disability in PPMS is caused by the diffused antibodies produced by B cells, which act as CNS antigen-presenting cells and trigger further T cell activation [17, 22, 23].

1.1.1.3 Secondary-progressive MS

About 50% of patients with RRMS develop secondary progressive MS (SPMS) within 10-15 years, until then, their symptoms persist without full remission [10]. SPMS is more complex and can be seen as a combination of RRMS and PPMS. Initially, it has a period of relapsing-remitting fluctuation, which is followed by a gradually worsening symptom [24]. SPMS can be considered as RRMS with insufficient time for remission and finally "deteriorated" to PPMS. SPMS keeps constant severity of disability in the first decades, which is then followed by a stepwise worsening period with seldom remission phases after 10 to 15 years. Similar to PPMS, SPMS also has prominent demyelination in the cerebral and cerebellar cortex resulting from the action of autoantibodies produced by B cells in the CNS.

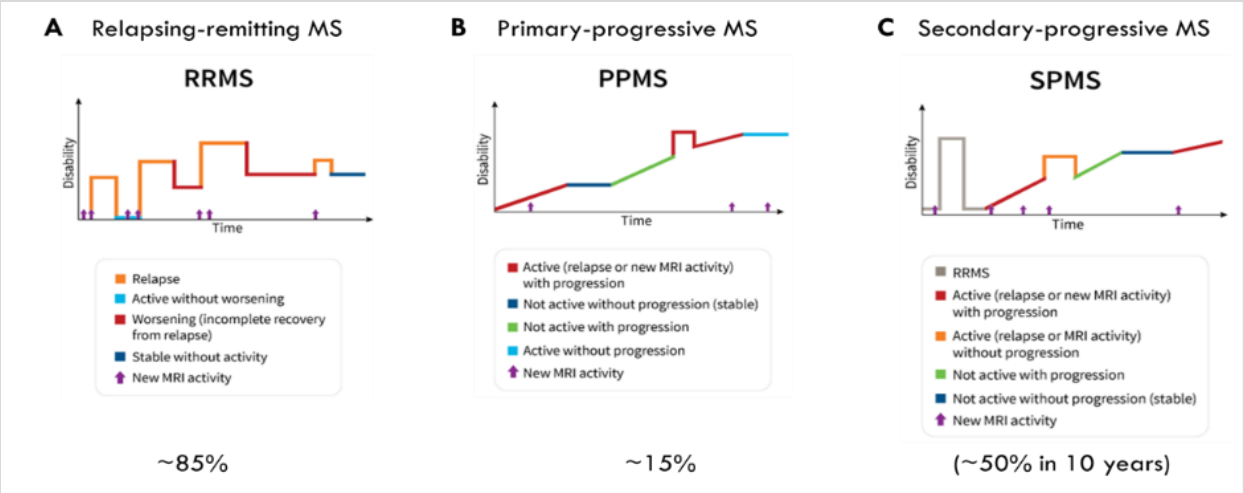

Figure 1.2: Clinical courses of multiple sclerosis (MS). (A) Relapsing-remitting MS (RRMS), which affects about 85% of the MS patients. (B) PPMS affects the rest of 15% of MS patients. (C) Up to 50% of RRMS patients develop SPMS within 10-15 years. [Ref: https://www.nationalmssociety.org/What-is-MS/Types-of-MS]. Used with permission from the copy right owner National Multiple Sclerosis Society.

1.1.2 Neuropsychiatric conditions in MS

Emotional disturbances can cause enormous suffering and significant disruption of one's family relationship, personal work, and social life [25]. Mood instabilities arising from demyelination can be used as a therapeutic indicator for treatment implications [26].

1.1.2.1 Anxiety

Anxiety is a natural response to potential danger and risk with a feeling of apprehension due to stress [27, 28]. It becomes a disorder when people suffer from chronic and functional impairing anxiety [29]. Anxiety disorder commonly affects information processing speed and working memory, thus directly impacting cognitive function [30, 31] [32, 33]. It is noteworthy that long-term anxiety is closely associated with emotional dysregulation, reduced life quality, and increased

suicide risk [34]. The lifetime prevalence rate of anxiety in MS is between 19.3% to 35.7%, which is significantly higher than in the general population (5%) [35-37].

1.1.2.2 Depression

Depression is a psychiatric condition that presents with a persistent and intense feeling of sadness, hopelessness, loss of interest, fatigue, or irritability lasting over two weeks [38]. The lifetime prevalence of depression in MS is approximately 50% [39, 40]. Studies suggest that frontal and temporal cortical atrophy, microglial activation, and WM and grey matter (GM) damage are all critical contributors to the development of depression in MS [41]. Depression increases suicidal risk, and significantly increases the morbidity and mortality of those with MS. The reported suicide risk peaks within five years after diagnosis, with over 50% of suicides occurring in this interval [42]. Further, the relative risk for contemplating suicide was highest within five years of diagnosis, and after more than 20 years of illness [42, 43]. Depression frequently co-occurs with cognitive impairment (CI) and adversely impacts cognitive function [44]. MS patients with CI tend to escape when facing a challenging problem [45], which leaves them vulnerable to depression and frequently emotional flooding leading to worsening of their condition.

1.1.2.3 Cognitive impairment

CI is one of the leading causes and the most significant determinant of MS disability that affects 40-65% of patients [46-49]. It can occur at any stage of the disease but is more frequent and more severe in PMS [50, 51]. CI affects family relationships, social communication, career development and mental health. The most common affected domains of cognition are attention, visuospatial abilities, information processing speed, executive functions, and learning and memory [49, 52, 53].

CI is challenging to detect in the early period or before MS onset [54]; however, as MS progresses, there is a constant deterioration of the cognitive functions.

Recent studies have confirmed that both GM and WM are involved in the pathogenesis of MS. Therefore, some clinical symptoms, like CI, can be explained better. Traditionally, MS is considered as a WM pathology in which inflammatory lesions and retrograde changes are the results of demyelination and cognitive function damage [55]. However, only moderate cases of WM demyelination are correlated with CI, suggesting that WM pathology alone is not enough to explain the mechanism of CI completely [56]. Different from WM lesions, GM lesions are non-inflammatory but can precede further WM pathology and are characterized by diffuse damage [57].

1.1.3 Neuropathological changes in MS

Previous research indicated that MS is a two-stage disease that starts with an active inflammatory phase and later transforming into a chronic and diffused neurodegenerative stage [58, 59]. Demyelination and neurodegeneration are two representative pathologies in PMS. Demyelination represents the damage of the myelin sheath that surrounds and protects axons. Neurodegeneration refers to the loss of function or death of the neuron.

1.1.3.1 Demyelination and myelin integrity

Myelin is a unique structure with high lipid content (~70%) and high enrichment of myelin basic protein (MBP) and proteolipid protein (PLP), which are the major component of CNS myelin [60]. In humans, around 40% of the brain contains WM, where myelin is the main component (50–60% dry weight of the WM) [61]. In the CNS, myelin is generated by OLs, and each OL form multiple branches (up to 30 or more) that interact with different axon bodies. In contrast, Schwann cells generate myelin in the peripheral nervous system (PNS), and each Schwann cell only builds a

single connection with a single axon body [62]. The establishment of a myelin sheath is comprised of three essential steps: (i&ii) wrapping and remodelling by OLs; and (iii) compaction by MBP [63]. The extent of myelin sheath formation is dynamically regulated by the plasticity to induced as brain function adapts to the environmental stimuli [64-66].

The myelin sheath is involved in neuroprotection and enables rapid action potential propagation via saltatory conduction across nodes of Ranvier through voltage-gated Na+ channels [67]. This increases the speed of conduction, reduces the energy, and decreases the reaction time of the organism. Therefore, axons that are fully myelinated along their length conduct impulses faster than unmyelinated axons of the same cross-sectional size [68].

Demyelination leads to damage in the protective covering myelin sheath that surrounds nerve fibres in CNS (brain) and PNS (optic nerves and spinal cord). Demyelinated axons are exposed to neurotoxic insults, oxidative stress, and energy deficiency, and are therefore vulnerable to further injury, which can result in irreversible axonal damage [69-72]. There are two main mechanisms of demyelination: Outside-In model, where peripheral immune cells (represented by T cells, B cells, and macrophages) are involved; and Inside-Out model, which involves oligodendrocyte demyelination and loss of mature oligodendrocyte [73, 74].

1.1.3.2 Oligodendrocyte maturation

Oligodendrocyte precursor cells (OPCs) are progenitor cells that can proliferate [75]. During remyelination, OPCs migrate to the demyelinated area and differentiate into OLs [76, 77]. The differentiated OLs form myelin sheaths around axons and support saltatory conduction of neural signals [76, 77].

OPCs respond to injury and promote recovery [78]. In chronic or severe acute demyelinated areas, where mature OLs are damaged or dead, OPCs are capable of differentiating into oligodendrocytes and reducing the impairment due to the loss of damaged oligodendrocytes. In response to myelin damage, OPCs are activated and recruited to demyelinated areas, and if driven to differentiate into OLs, can help to reconstruct a new myelin sheath [79]. This regenerative process induced by OPCs is called remyelination where OPCs undergo rapid proliferation, migration, and directional differentiation [80]. However, impairment of myelin debris clearance inhibits OPCs from differentiating into mature OLs [79, 81]. Therefore, the accumulation of myelin debris and OPCs also leads to incomplete remyelination, which leaves lesions which are more vulnerable to inflammatory damages targeted at the axon and OL. Glutathione S-transferase (GST)-pi is a cytosolic isoenzyme, which previously found to be associated with oligodendrocyte maturation and myelin sheath formation [82, 83]. In addition, GST-pi plays an important role in detoxification of harmful compounds by catalyzing glutathione conjugation [84]. Therefore, GST-pi can be used as a stage-specific maker for mature myelinating oligodendrocyte and a marker for the detoxification [85, 86]. Other markers, like NG2 and GPR17, are required to detect early OPCs and immature oligodendrocytes, thus completing the fate-tracking analysis of oligodendrocyte lineage [85].

The pathological features vary among the different courses of MS. RRMS is characterized by active lesions, focal inflammatory WM lesions, inflammation-dependent neuronal damages, and active remyelinated remissions [87, 88]. PMS has chronic diffused inflammation in both WM and GM, with more prominent cortical demyelination, and impaired remyelination [89].

1.1.3.3 Microglia activation and polarization in MS

Microglia are resident immune cells in the CNS that play critical and dynamic roles in MS pathology. In response to inflammation and demyelination, a large proportion of resting microglia polarize into pro-inflammatory M1 or pro-repair M2 phenotypes, which is a result of reactive phenotypic transformation [90]. Rapid and massive expression of pro-inflammatory cytokines can further stimulate microglia activation [91]. Studies in animal models have shown that M1 and M2 microglia play distinctive roles in MS progression [91, 92]. M1 microglia dominate the demyelination phase, while M2 microglia are prominent during the remyelination process [91, 92]. Classically, activated M1 microglia have the properties with neurotoxicity and pro-inflammation [93, 94]. Immunohistochemistry staining using M1 markers (tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and nitric oxide (NO)) showed abundant M1 microglia in the active, destructive demyelinated lesions [95-97]. These neuroinflammatory factors generate excitotoxicity and mitochondrial dysfunction that leads to neurodegeneration and OL damage [70-72].

In contrast, M2 microglia are considered neuroprotective and anti-inflammatory [98]. M2 microglia are involved in tissue repair by producing anti-inflammatory cytokines and factors promoting growth [92]. M2 microglia facilitate remyelination by cleaning up collapsed myelin debris and apoptotic cells in the demyelinated areas through phagocytosis [99-101]. M2 microglia also release anti-inflammatory factors ((e.g., IL-10, transforming growth factor-beta (TGF-β), and glucocorticoids)) to promote neurogenesis, OPC recruitment and remyelination [98, 102]. M2 microglia abnormality is strongly related to CI, emotional disturbances, and incomplete remyelination [103-105]. The depletion of M2 microglia inhibits remyelination, thereby interfering with the CNS recovery after injury [106]. M2 microglia abnormality also occurs in other neuropsychiatric diseases, including Alzheimer's disease, epilepsy, and major depressive disorder [103, 107, 108].

In summary, microglia activation has both beneficial and detrimental roles on CNS regeneration [109]. Activation of M1 microglia releases destructive pro-inflammatory factors that can precede inflammation and demyelination. The shift in the microglia from an M1 to protective M2 phenotype can serve to prevent chronic demyelination and axonal injury, as well as improving cognitive and emotional symptoms [110-112].

1.1.3.4 Remyelination in MS

Remyelination is a spontaneous regenerative process identified with the production of new myelin sheaths around demyelinated axons [113]. Remyelination is a frequent restorative event in the early stage of RRMS [114]. In chronic lesions of PMS, remyelination becomes inadequate and eventually aborts with the disease progression and ageing [115]. The impairments of OPCs are presumed to take major responsibility for the delay or failure of the remyelination process, which includes the failure of OPC recruitment or failure to differentiate into mature OLs [116, 117].

Newly remyelinated tissue is vulnerable to inflammation and incomplete or failed remyelination, is also evident after repeated MS attacks [118]. Finally, there are only 10–20% of the chronic lesions, which can be completely remyelinated [119]. It is noteworthy that the expressions of growth factors, including TGF-β, are associated with remyelination, with delayed expressions of growth factors correlate with slowed remyelination [120]. Specifically, a decrease in TGF-β expression prevents remyelination in the spinal cord after toxin-induced demyelination [121].

1.2 Transforming growth factor-β pathways

1.2.1 Signal transduction

TGF-β represents a large family of multifunctional growth factors that are critical for regulating various biological processes such as embryonic development, immune response, and cellular proliferation and differentiation [122]. TGF-β also acts as a growth inhibitor on epithelial cell or endothelial cell proliferation [123].

There are three types of TGF-β receptors (TGF-β-RI, RII and RIII). These receptors have distinctive structures and functions in modulating the ligand-binding and in regulating the TGF-β expression on the surface of the cells [124, 125].

In this study, we mainly focussed on TGF-β-RI and TGF-β-RII because of their prominence in signal transduction. The TGF-β pathway can be directly activated when TGF-β binds to a heterotetrameric TGF-β receptor complex composed of two RIs and two RIIs [126]. In the complex, TGF-β induces RIIs to phosphorylate and activate the RIs, which phosphorylates the C-terminal serine of Smad2 and Smad3 [127]. The phosphorylation-activated Smads (also named R-Smads) then activate the common-mediator (Co) Smad (also named Smad4), forming a trimer consisting of two R-Smads and one Smad4 [126, 128]. Finally, the trimer translocates to the nucleus, regulating gene expression [129, 130] (Figure 1.3).

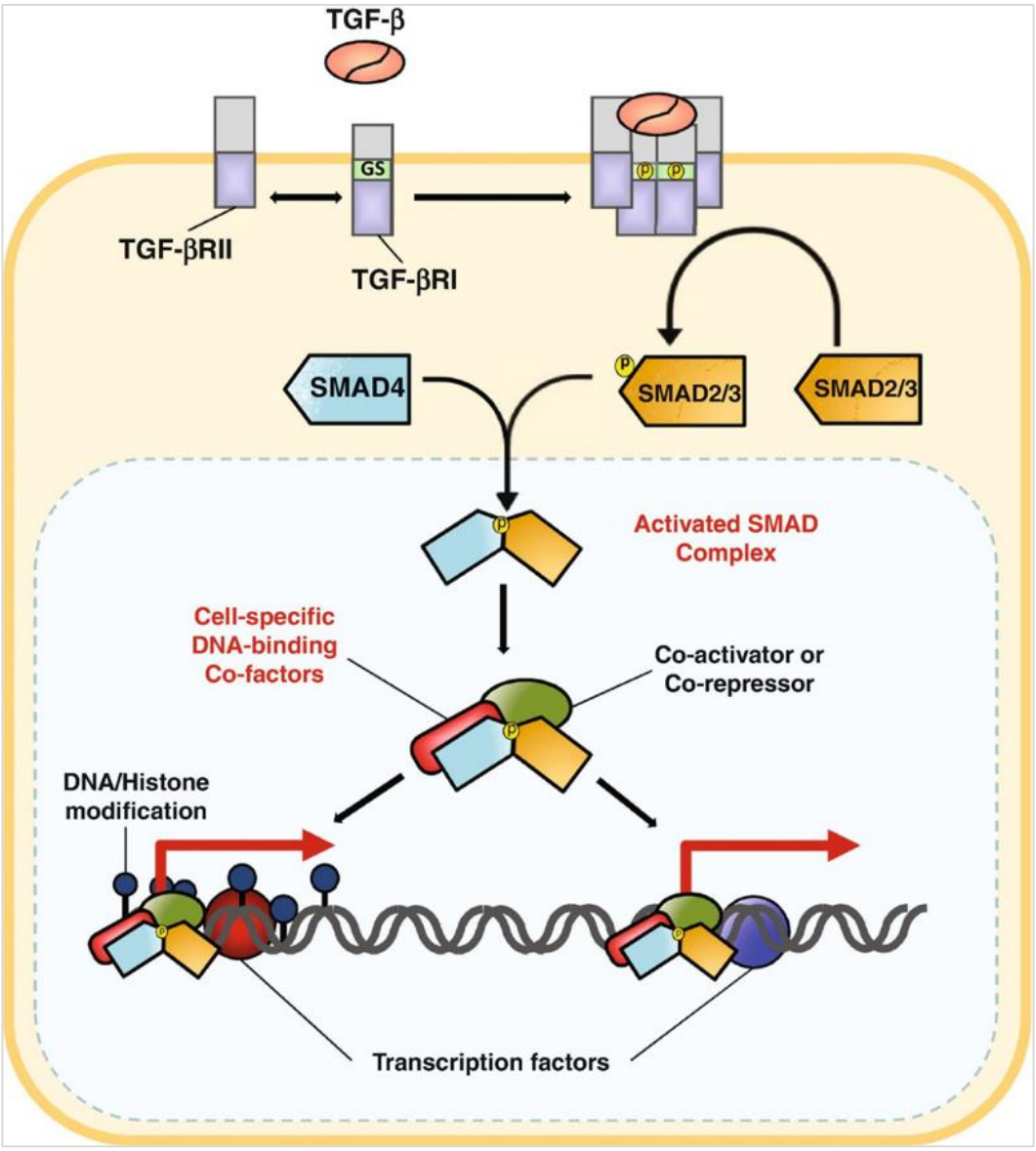

Figure 1.3: The TGF-β signalling pathway. Transforming growth factor (TGF-β) binds to the Type II receptor and recruits Type I. The Type I receptor, which is phosphorylated and activated by Type II receptor, in turn, phosphorylates Smads (Smad2/3) transcription factors. Smad4 helps activated Smads to translocate into the nucleus upon cellular stimulation. [Ref: Greenwood, W. and A. Bruna, TGF-β and the SMAD Signaling Pathway in Carcinogenesis in Predictive Biomarkers in Oncology. 2019, Springer. p. 305-310.]. Used with permission from the copy right owner Springer Nature.

1.2.2 Transforming growth factor-β pathways and MS

Previous studies have shown an involvement of the TGF-β pathway in MS. The results indicate that TGF-β regulates CNS myelination by modulating OPCs differentiation, with a decrease in TGF-β expression preventing remyelination in the spinal cord after toxin-induced demyelination [121, 131]. In the present study, we aimed to determine whether LFMS regulates the expression of TGF-β which may serve to promote remyelination after chronic cuprizone-induced demyelination.

TGF-β is a crucial regulator of cell proliferation, migration, differentiation, survival, and microglia polarization (from M1 to M2) [122]. TGF-β was identified as an anti-inflammatory factor that inhibits the production of reactive oxygen species (ROS) by activated microglia [98, 102, 132]. TGF-β receptor knock-out (KO) in the transgenic mouse model was shown to prevent CI and the disruption of the blood-brain barrier (BBB) in epilepsy [133]. In addition, TGF-β signalling plays a vital role in the process of regulating regulatory T cell (Treg) development and normal function [134]. Treg dysregulation suppresses immune responses in inflammatory sites, thus the deficit in Treg expression or function is commonly associated with autoimmune diseases, including MS [135].

1.2.2.1 Magnetic stimulation as a potential treatment for MS

In addition to therapeutic or immune-modulating drugs, there are also some existing novel treatment techniques for MS management, including repetitive Transcranial Magnetic Stimulation (rTMS). rTMS is a focal, non-invasive brain stimulation technique with limited side effects. There are twice transductions between a magnetic signal and an electrical signal (Figure 1.4). First, an electromagnetic coil on the scalp creates an area with magnetic pulses, which are single-cycle sine

pulses with a period of about 0.28ms at 1–20 Hz [136, 137]. Then, these magnetic pulses penetrate the brain without breaching the brain surface (e.g., craniotomy) [138]. Upon encountering nerve cells, magnetic energy transduces back to electrical energy, thereby affecting the neural tissue by regulating the electrical current to flow.

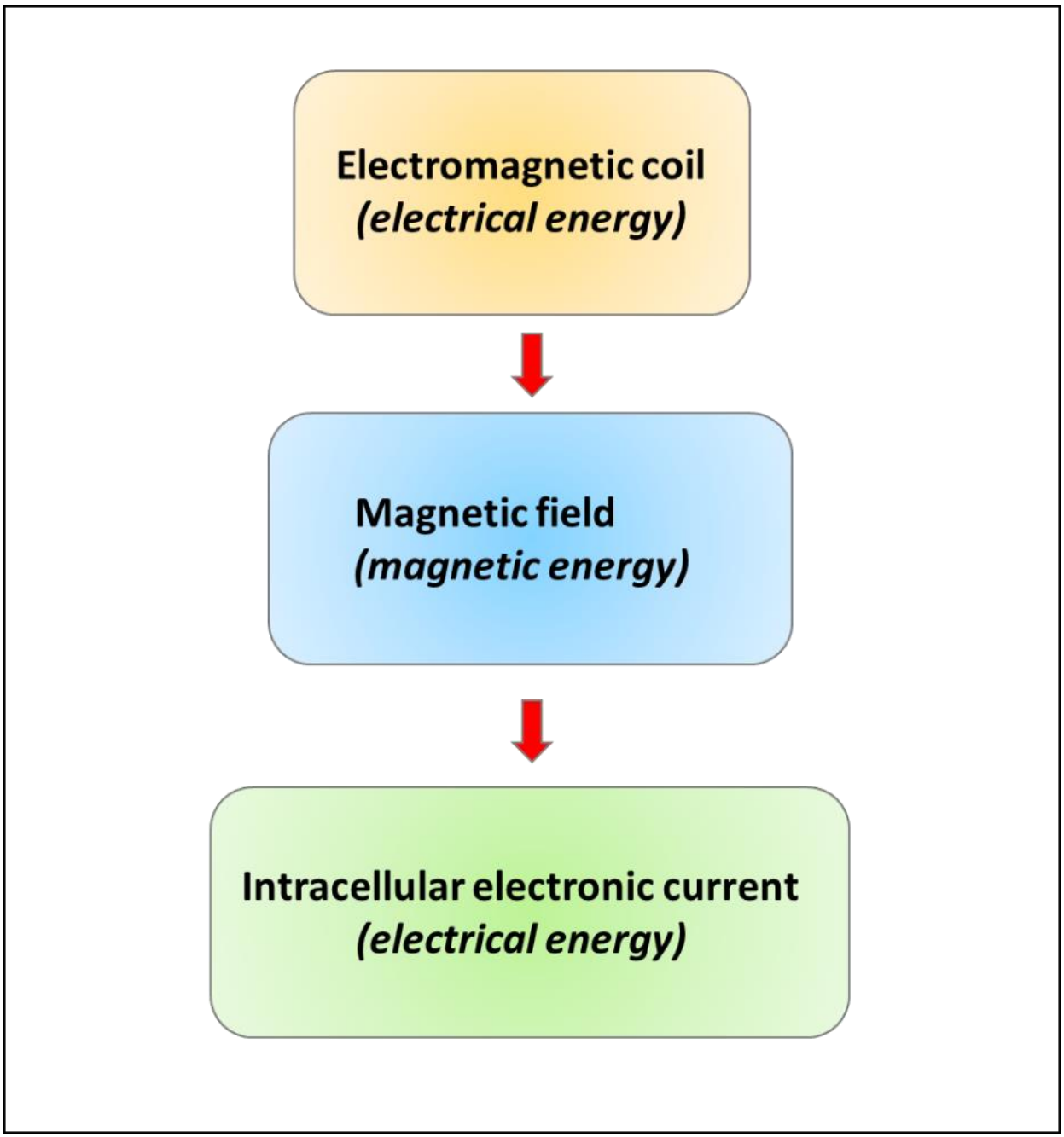

Figure 1.4: The electrical and magnetic signalling transduction of repetitive Transcranial Magnetic Stimulation. Electronic energy produces a magnetic field, which penetrates the skull to deep brain regions, where the magnetic energy encounters nerve cells and transduces back to electrical energy, thus completing the signalling transduction and stimulating brain regions

1.3 Low Field Magnetic Stimulation

Low Field Magnetic Stimulation (LFMS) is an experimental form of non-invasive neurostimulation device that produces diffuse, low-intensity (≤1 V/m, 1kHz), and oscillating magnetic stimuli to multiple cortical areas [139, 140]. It aims to use low field strength magnetic stimulation to manipulate brain function. The magnetic field changed between uniform and linear gradients (Figure 1.4). Each gradient is composed of several on or off cycles (Figure 1.5). Clinically, LFMS has shown beneficial effects in the treatment of mood disorders, including bipolar depression and major depressive disorders [140, 141], in maintaining synaptic plasticity and brain connectivity [142], and in improving cognitive impairment in neuropsychiatric disorders, such as Alzheimer's Disease, Schizophrenia, and Post-traumatic Stress Disorder [143-145].

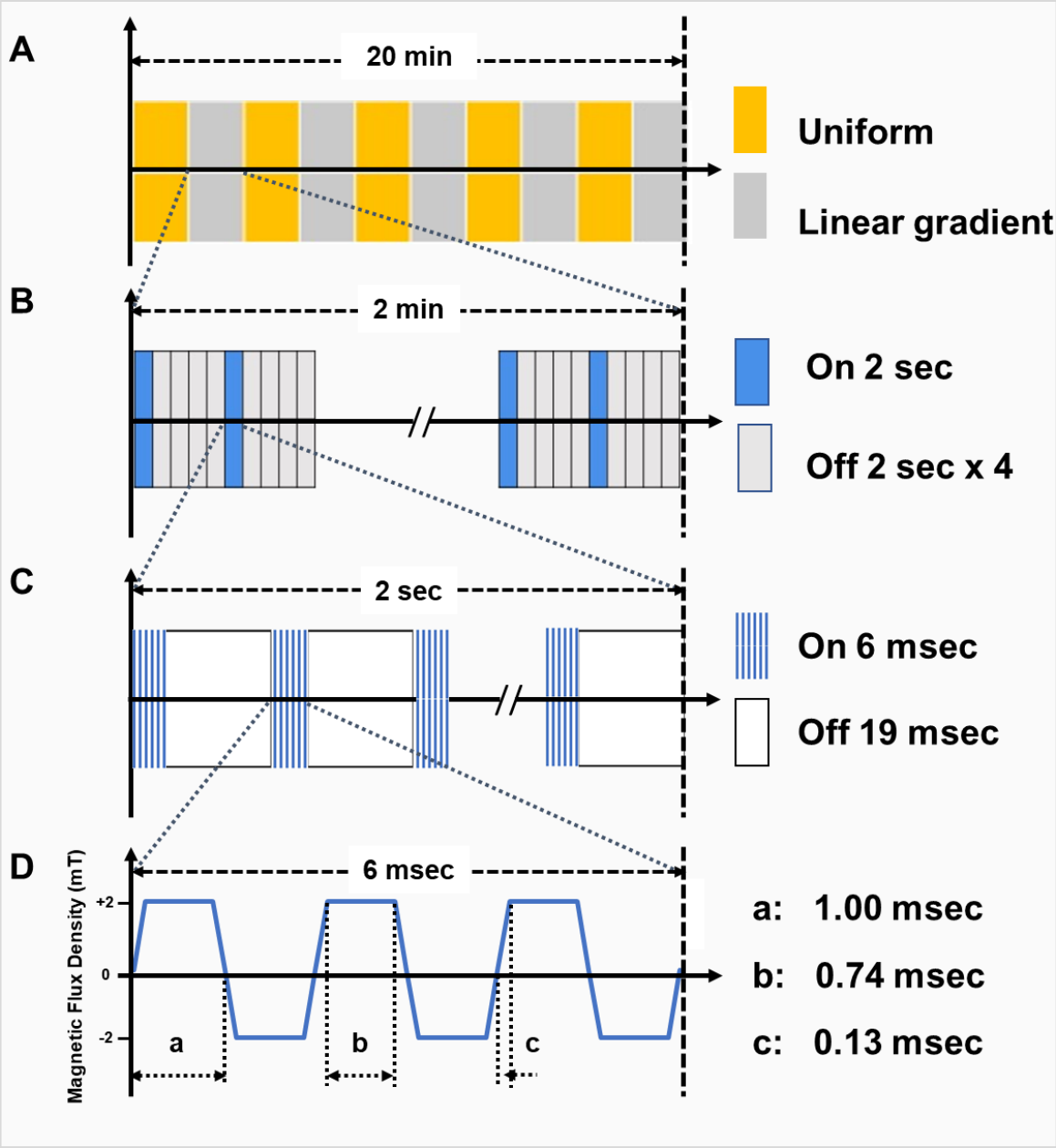

Figure 1.5: The gradients with on or off cycles in either gradient. (A) The magnetic field changed every 2 min between uniform and linear gradients. (B) Each cycle consisted of 2 seconds (sec) on and 8 sec off. (C) Each 2-second stimulation was composed of rhythmical trains, which has 6 pulses at 1000 Hz frequency and 19 msec intervals. (D) The Maximal magnetic flux density (BMax) is less than 2 mT, and the peak induced electric field (EMax) is less than 0.5 V/m.

1.3.1 Mechanism of action of LFMS on remyelination

In our current study, we aimed to determine whether LFMS treatment could decrease CPZ-induced cognitive impairment, demyelination, and astrocyte activation in mouse brains. Some pieces of evidence indicated that OL functions (OPC proliferation, migration, and differentiation) could be controlled by glutamate and GABAergic pathways [146, 147]. Therefore, LFMS might affect the signal transduction in the neuron-glial interaction, thereby leading to remyelination and OL lineage development. Base on this, we propose that LFMS has therapeutic potential for PMS by regulating microglia function, promoting OL differentiation, and enhancing remyelination.

1.3.2 Advantages of Low Field Magnetic Stimulation

LFMS is beneficial due to the following effects: i) rapid mood improvement after one brief treatment; ii) completely non-invasive treatment, free from painful feeling and side effects; iii) portable since it is the size of a regular laptop, which allows home use and the ease of operation; iv) low intensity but deep regional neurostimulation; and v) relatively lower cost compared to other instruments [140].

2 HYPOTHESIS AND OBJECTIVES

Previous studies have shown that cognitive deficits were improved by LFMS[148]. We hypothesize that LFMS can alleviate demyelination-related cognitive deficits and depression-like behaviour in a chronic Cuprizone (CPZ) mouse model of MS by promoting remyelination. The objectives of the study are: 1) To determine the effects of LFMS on cognitive decline and mood disturbance; 2) To determine the effects of LFMS on remyelination and OL recovery. Based on recent reports about the anti-inflammatory actions of TGF-β described above, we chose to analyze the

role of the TGF-β signalling pathway as a possible mediator of the effects of low-field magnetic stimulation (LFMS) treatment in remyelination. We anticipated identifying the signalling molecules in the TGF-β pathway that are responding to the LFMS treatment during the remyelination process. The goal was to confirm whether the remedial LFMS leads to remyelination in the chronic cuprizone treated mice, which were demyelinated.

3 MATERIALS AND METHODS

3.1 Cuprizone-induced chronic demyelination model

3.1.1 Introduction

Several animal models have been developed for studying MS, which are represented under three broad categories, namely: i) experimental autoimmune encephalomyelitis (EAE); ii) cuprizone intoxication; iii) Theiler's murine encephalomyelitis virus (TMEV) infection [149, 150]. Each model has its unique values and limitations in MS research.

In this study, we utilize the cuprizone-induced chronic demyelination model (Figure 3.1 [151]). Cuprizone (CPZ) is a copper chelator used to generate CNS demyelination and OLs depletion [152, 153]. CPZ-induced toxic model exhibits the advantage that CPZ generates inside-out and non-inflammatory neuronal damages without involving peripheral immune cells [57], thus providing a suitable environment to explore the mechanism and interactions of de- and remyelination [154]. Because CPZ model studies oligodendrocyte apoptosis and subsequent demyelination, CPZ model shows a drawback that it does not readily mimic the clinical progress of MS, which involves peripheral immune responses, characterized by the reactions of T cells, B cells, and macrophages

[57]. In addition, the variability of the CPZ consumption is another disadvantage during the study, which will differ from one mouse to the other [155].

An acute exposure (for 5–6 weeks) of young adult mice to 0.2% CPZ causes diffused lesions (significant myelin depletion, OPC proliferation, and microglia activation) in both GM and WM areas, which is represented in the prefrontal cortex, hippocampus, cerebellar peduncles, and corpus callosum [156]. Spontaneous but incomplete remyelination occurs within weeks after removal of CPZ from the mouse diet, with the effects of CPZ administration being partially reversed [157].

Prolonged exposure to CPZ (up to12 weeks) leads to progressive demyelination and irreversible axonal damage, followed by incomplete remyelination and chronic microglial activation [158]. Chronic CPZ feeding can effectively exhaust mature OLs population and reduce their ability to sustain existing myelin and generate new myelin [153]. These selective targeting attacks make CPZ model become a suitable model to study OL apoptosis and myelin depletion [159, 160].

The mechanism of CPZ-induced OL death remains unclear. Previous studies have shown that CPZ may cause a copper deficit that leads to oxidative stress and mitochondrial dysfunction. During the peak of myelin formation, oligodendrocytes generates three times of their weight in membrane, which requires a high cellular metabolism and a large amount of ATP [161]. Dysfunctional mitochondria ultimately do not allow the cells to meet their metabolic needs, which eventually results in apoptosis [162]. CPZ also triggers microglial activation and pro-inflammatory cytokine release, which further aggravates OL and axonal damage [157].

In addition, characterized by targeting mature OL, the CPZ model provides a suitable model to study the neuropsychiatric mechanism of MS symptoms and promote novel interventions for MS

treatment by avoiding adaptive immune system activation and blood-brain barrier (BBB) break down [163].

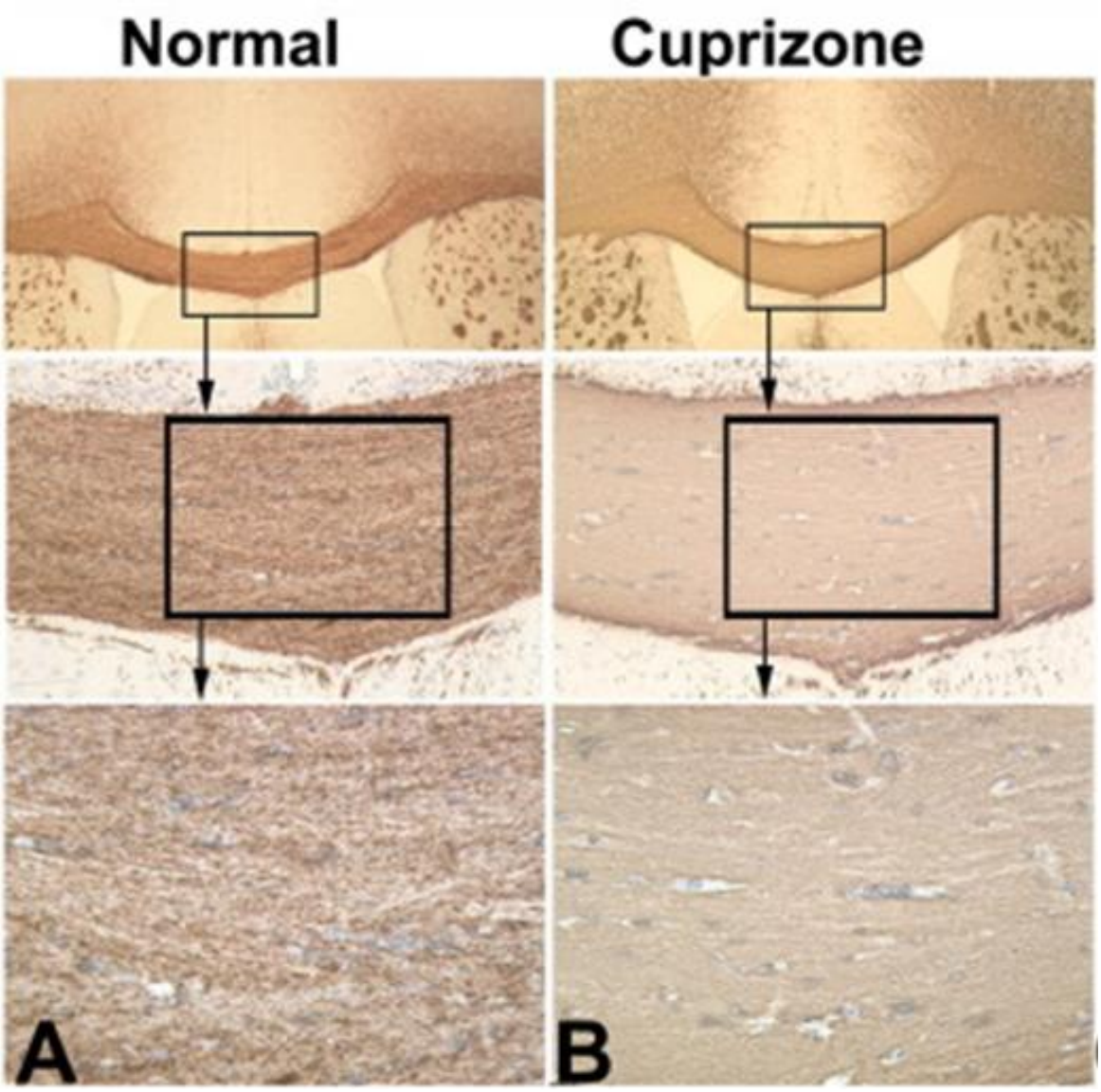

Figure 3.1: Demyelination in the corpus callosum of cuprizone treated mice. (A) Immunostaining of MBP in the corpus callosum of healthy mice. (B)A significantly decreased MBP level in the corpus callosum after five weeks of CPZ exposure was revealed by immunostaining. [Ref: Zhang, J. et al., Thymosin beta4 promotes oligodendrogenesis in the demyelinating central nervous system. Neurobiology of Disease, 2016. 88: p. 85-95.]. Used with permission from the copy right owner Springer Nature.

3.1.2 Animal modelling

C57BL/6 mice (8 weeks old, female) were randomly assigned into three groups, consisting of control (CTL) mice, and CPZ-treated mice with or without LFMS exposure. Specifically, the CPZ group consisted of two groups, with one receiving Sham treatment (CPZ) and with the other receiving LFMS treatment (CPZT). A total of 70 mice were used for this study, the number of animal used in each treatment group were calculated by power analysis and type I error [164, 165]. CTL mice were fed with the regular powder diet for 16 weeks. CPZ mice were fed with 0.2% of CPZ in powder diet (w/w) for 12 weeks (12w) to induce chronic brain demyelination, which was followed by a regular diet for four weeks. The food intake was monitored by controlling the amount given to animals between animal diet refills. Following chronic and toxic CPZ exposure, mice display 'sickness' behaviour that is characterized by weight loss [159]. Thus, the body weights of mice were used as a clinical indicator to evaluate the CPZ-induced demyelination severity. Mice were weighed individually at the beginning of the studies, during CPZ administration, and before sacrifice. During CPZ administration, the body weights of mice were taken and recorded each week. The effects of LFMS on remyelination and cognitive improvement were assessed when the CPZ mice returned to a regular diet for 2 and 4 weeks (14w and 16w of the entire experiment).

3.2 Low Field Magnetic Stimulation treatment

The metal lid of the cage was removed before the cage was placed on the LFMS device (Figure 3.2). Mice in LFMS group received a 20-min LFMS treatment daily for five days a week. In comparison, mice in the Sham group received no treatment after 12 week cuprizone diet. This was completed on the LFMs machine for 20-min treatment without the application of LFMS stimulus parameters. The 40 Hz LFMS settings were based on previous studies with minor modifications

[166, 167]. Briefly, the magnetic field changed every 2 min between uniform and linear gradients. Each cycle consisted of 2 seconds (sec) on and 8 sec off [166, 167]. Each 2-second stimulation was composed of rhythmical trains, which has 6 pulses at 1000 Hz frequency and 19 msec intervals [167]. The Maximal magnetic flux density (B_{Max}) is less than 2 mT, and the peak induced electric field (E_{Max}) is less than 0.5 V/m [166] (see Figure 3.2). Animals in the sham group went through the same treatment routine, but with no magnetic stimulation. The treatments lasted for two (14w) or four weeks (16w). The effects of LFMS were studied on the locomotor functions, anxiety and depression-like symptoms, as well as working memory using behavioural tests as discussed later.

Figure 3.2: Low Field Magnetic Stimulation treatment. Low Field Magnetic Stimulation treatment was implemented for 20-min. Mice were placed in the cages, and the lid of the cages was removed during the treatment. Animals in the sham group were treated similarly, but without magnetic stimulation.

3.3 Behavioural Tests

3.3.1 Open Field Test

Open Field Test (OFT) is a behavioural test that involves a conflict between the desire to explore and the desire to avoid the anxiogenic stimuli of open space in rodents [168]. In this study, OFT was used to examine the gross locomotor function and anxiety-like behaviours of the mice. The total distance travelled indicates an animal's gross locomotor function, and the duration spent in the center area of the box was used to evaluate the anxiety level of the tested animal [169]. Each mouse was placed at the center of a box (42cm*42cm*40cm high) and allowed to explore the area freely for 5 mins. The floor of the box was virtually divided into 16 identical squares on ANYmaze software. The 12 outer squares along the wall were identified as the peripheral area; the 4 central squares were identified as the central area (Figure 3.3).

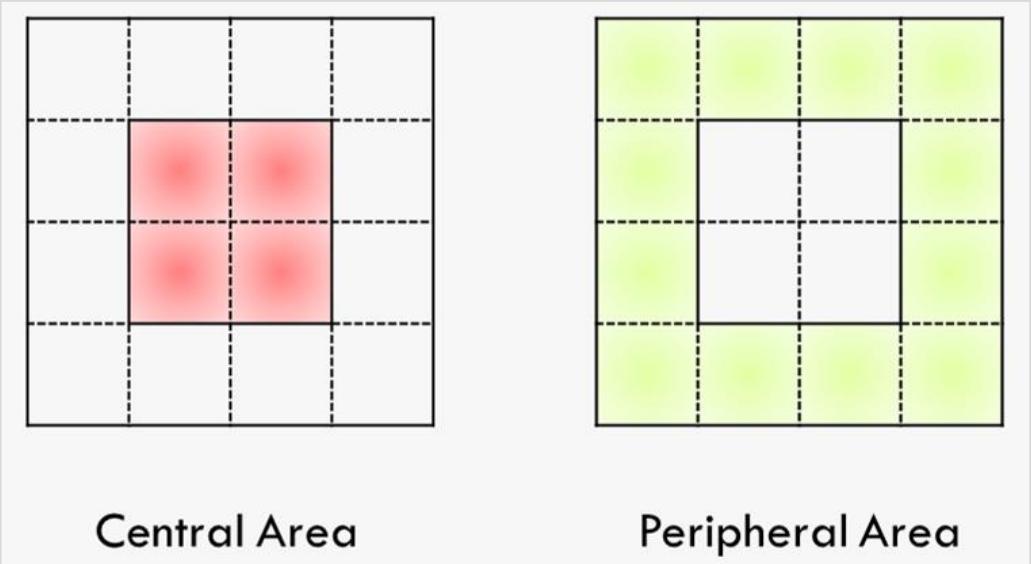

Figure 3.3: Open Field Test (OFT) arena. In our study, the testing surface of OFT was 42 cm*42 cm and was subdivided into 16 equal squares (4 by 4 matrix) for locomotor and anxiety-like behavioural data acquisition.

3.3.2 Y-maze

Y-maze (Figure 3.4) is a behavioural test that is extensively used to evaluate working memory. The percentage of the spontaneous alternations completed during the 5-min test period were recorded and analyzed. Y-maze test examines if the mice remember the arm they have just explored and therefore enter two other previously unexplored arms of the maze [170]. Mice were placed in the center of the maze and allowed to explore all three arms freely. Each time that a mouse completed a set of three non-repeating entries was recognized as one spontaneous alternation.

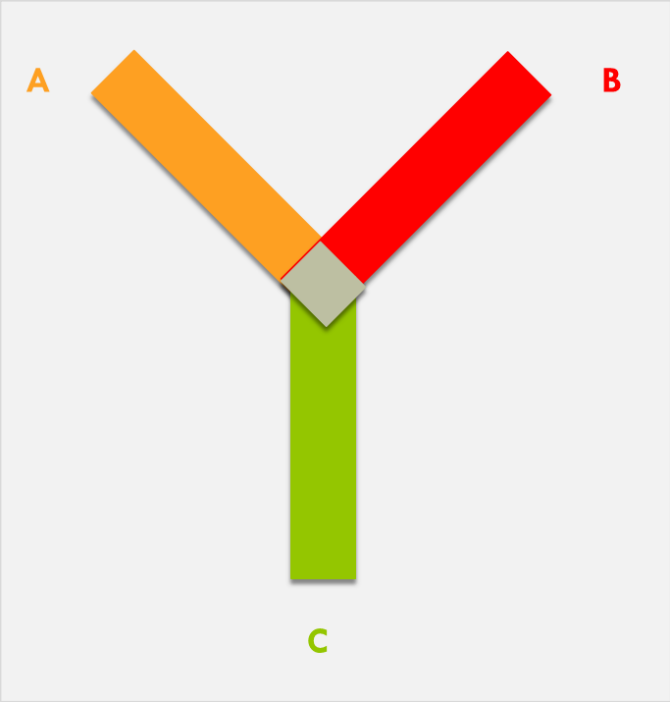

Figure 3.4: Y-maze arena. In our study, the arms were labelled as A, B, and C, clockwise. Each time that a mouse completed a set of three non-repeating entries was recognized as one spontaneous alternation.

3.3.3 Tail Suspension Test

Tail Suspension Test (TST) is a behavioural test that induces "behavioural despair" that animals give up the attempts to escape in an inescapable situation [171]. The mice were suspended on the edge of a shelf, 58 cm above a tabletop by adhesive tape for 6 min. The immobile time in the last 4 mins of the test was measured as behavioural despair and the depressive state of the animal [172]. In the study, we divided the tail suspension box (120cm W x12cm D x 60cm H) into four compartments (28cm W x 12cm D x 60cm H). Each mouse was suspended within its compartment to prevent the mice from seeing each other (Figure 3.5).

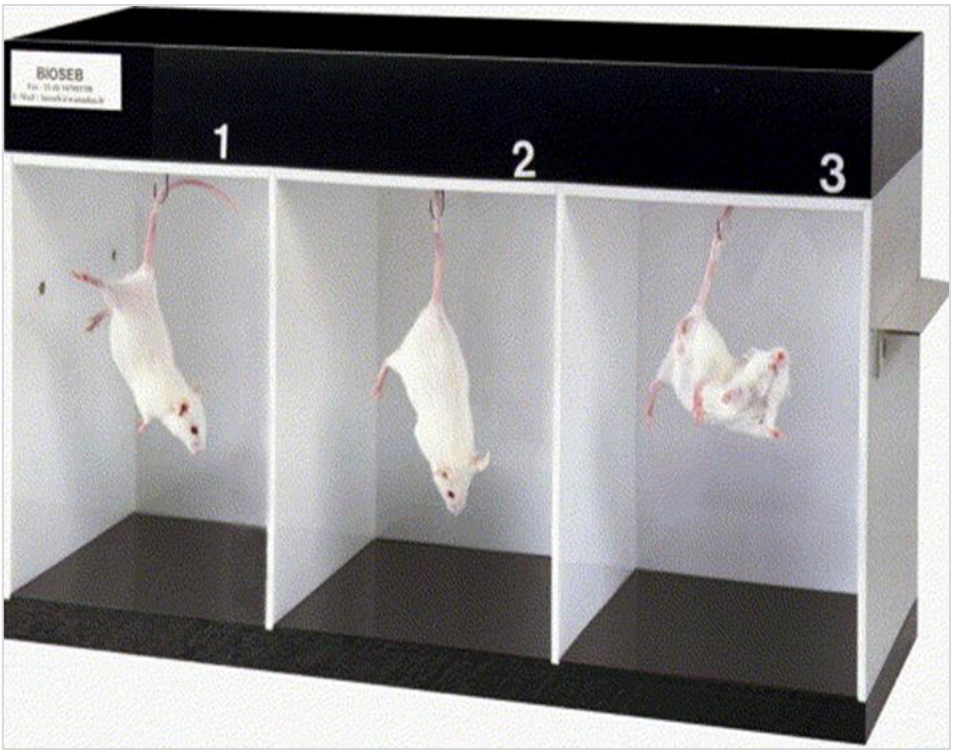

Figure 3.5: Tail Suspension Test (TST) arena. In our study, the shelf was designed as 58 cm height and 84cm in length; in such an arena, the mice could not escape or hold any nearby surfaces. Used with permission from the copy right owner Springer Nature.

3.4 Biochemical and pathological tests

After the completion of behavioural tests, the neural tissue was collected after euthanizing the animals, and stored at -80^{o}C for future experiments (Western Blots and immunohistochemistry).

3.4.1 Brain tissue collection

At each time point (12w, 14w, and 16w), 10 mice from each group were randomly selected for tissue harvest. Four mice were perfused with one-time Phosphate Buffered Saline (1x PBS), followed by 4% neutralized buffered paraformaldehyde (4% PFA) fixation. The harvested whole brains were preserved for future brain histology and immunostaining studies. The rest of the brains were perfused with 1x PBS for use in Western Blotting (WBs) and further dissected, thereby collecting the following regions: the prefrontal cortex (PFC), hippocampus, and striatum.

3.4.2 Sample preparation and SDS-PAGE

In this study, biochemical and pathological tests focused on the PFC, a brain area critical in emotional regulation and learning and memory [173, 174]. The PFC samples were lysed using RIPA lysis buffer mixed with protease inhibitor cocktail (1x PIC) and homogenized by LabGEN 125 and 700tissue homogenizers (Cole-Parmer, Montreal, QC, Canada). The homogenized samples were centrifugated at 4^{o}C for 10 mins at 12000rpm, and the supernatant was collected for determination of protein concentration. Bradford Protein Assay kit (Bio-Rad, Hercules, CA) and SpectraMax M5 multi-mode microplate reader (Molecular Device, San Jose, CA) were used to measuring the absorbance at 595nm and quantify protein concentration. The protein samples were mixed with the loading buffer at 90^{o}C for 5 mins before Western blotting.

Thirty-micrograms of protein from each sample were loaded and separated on 4-20% SDS-PAGE (4-20% Precast Gradient Protein Gel, Bio-Rad), and transferred onto a 0.45mm nitrocellulose membrane. The blocking step was performed in 5% (w/v) non-fat dry milk in 1x PBS with 0.1% Tween 20 (v/v) (PBST). The membranes were incubated in 5% (w/v) Bovine Serum Albumin (BSA) in PBST.

3.4.3 Antibodies

Rabbit anti-MBP (1:1,000; 78896s, Cell Signaling, Danvers, MA) and mouse anti-MOG (1:1,000; NB300-948, Novus Biologicals, Oakville, ON, Canada) were used to detect myelin sheath integrity. Rabbit anti-GFAP (1:1,000; HPA056030, Sigma Aldrich, St. Louis, MO) and rabbit anti-TNFα (1:1,000; ab9635, Abcam, Cambridge, UK) were used as a marker for astrogliosis and microglia activation. Rabbit anti-Olig-2 (1:1,000; P21954, Thermo Fisher Scientific, Waltham, MA) and rabbit anti-GST-π (1:1,000; ADI-MSA-102-E, Enzo Life Sciences, New York, NY) were used to identify the OL lineage and mature OLs. The combination of GST-π and Olig-2 enabled to investigate the changes of OPCs. Rabbit anti-TGF-β1 (1:1,000; ab92486, Abcam, Cambridge, UK), rabbit anti-TGF-β-R1 (1:1,000; ab135814, Abcam, Cambridge, UK), and rabbit anti-TGF-β-R2 (1:1,000; ab186838, Abcam, Cambridge, UK) were used to identify TGF-β pathway. Mouse anti-β-actin (1:1,000; a3854, Sigma Aldrich, St. Louis, MO) was used as a loading control. The secondary antibodies used were anti-rabbit fluorescent-conjugated secondary antibody (1:1,000, 926-32211, LI-COR Biosciences, Lincoln, NE), and anti-mouse fluorescent-conjugated secondary antibody (1:1,000; 926-68070, LI-COR Biosciences, Lincoln, NE).

3.4.4 Image Analysis

The intensity measurements after the WBs were completed by fluorescence detection using the scanner from LI-COR Odyssey 9120 imaging system (LI-COR Biosciences, Lincoln, NE). The analysis was completed using Image Studio software (Ver 5.2), and the values were recorded for the intensity of each band for future statistical analysis.

3.5 Data analysis

Statistical significance was evaluated by unpaired t-tests when applicable. The statistical significance cut off was set at $p < 0.05$. Data are shown as means ± SEM in figures and text. GraphPad PRISM software (Ver 8.0, GraphPad Software, San Diego, CA) was used to generate the graphs and perform the statistical analyses.

3.6 Ethical approval

This study used a well-established animal model. There were no human participants in the project. The LFMS treatment by itself does not cause any potential risk for the animal and researcher. Animal protocol (AUP20160103) was approved by the University Animal Care Committee (UACC) of the University of Saskatchewan, according to the Guidelines of the Canadian Council on Animal Care (CCAC).

4 RESULTS

4.1 Behavioural tests

4.1.1 Unimpaired locomotion in mice with cuprizone feed

Locomotor Function: The total distance travelled in OFT was used as a measurement of gross locomotor function. Unpaired t-tests found no significant differences in travel distance between CTL and CPZ groups after 12-week demyelination ($P>0.05$; Figure 4.1A) or between sham and LFMS treatment groups during remyelination ($P>0.05$; Figure 4.1B, C). In summary, neither CPZ nor LFMS treatment affected the gross locomotor function in OFT.

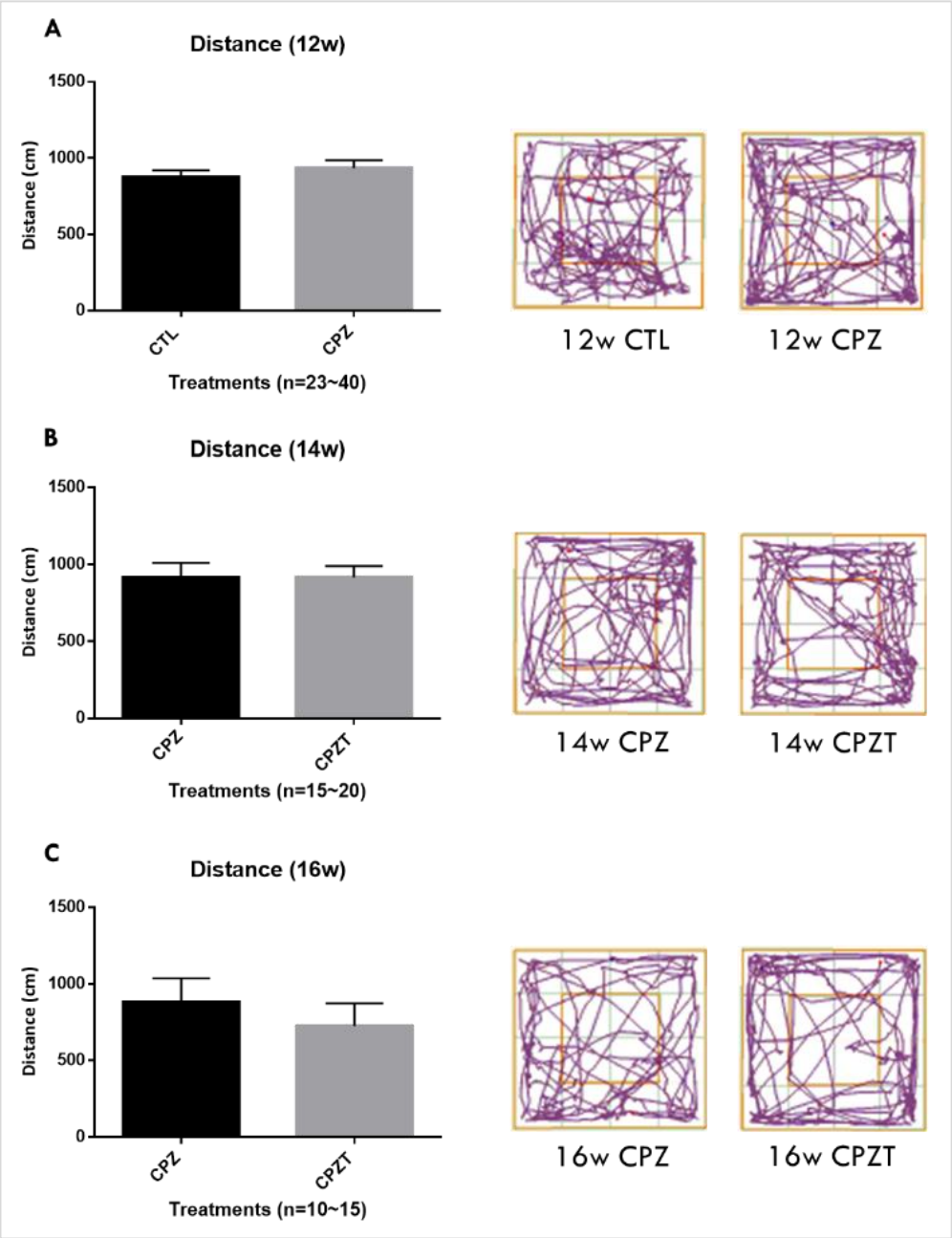

Figure 4.1: The effect of CPZ on locomotor function. (A) The total travelled distance in the control and demyelinated mice at the end of twelve weeks of CPZ feeding (n=23~40 per group). (B) The total travelled distance in the Sham (CPZ) and LFMS treated (CPZT) mice after two weeks of remyelination and CPZ withdrawal (n=15~20 per group). (C) The total travelled distance in the Sham (CPZ) and LFMS treated (CPZT) mice after four weeks of remyelination and CPZ withdrawal (n=10~15 per group). Data are expressed as means ± SEM.

4.1.2 Increased awareness of facing danger with LFMS treatment

Anxiety-like Behaviour: OFT also examines the level of anxiety; the time and distance spent in the central area of OFT were used to measure anxiety-like behaviour. Unpaired t-tests results showed no significant difference in central time and distance between the CTL and CPZ mice after 12 weeks of CPZ feeding ($P>0.05$; Figure 4.2A, D). The CPZT mice showed a trend of reduction in time and distance spent in the central area than the CPZ mice at 14w, which was conducted after two weeks of LFMS treatment ($P>0.05$; Figure 4.2B, E). The CPZT mice showed a significantly shorter time, and distance spent in the central area when compared to the CPZ mice after four weeks LFMS treatment (16w) (*$P<0.05$, **$P<0.01$; Figure 4.2C, F). Thus, OFT results revealed a significant reduction in the distance and time spent in the central area after treatment with LFMS for four weeks (16w), as shown in the panels 4.2C and 4.2F, respectively (Figure 4.2).

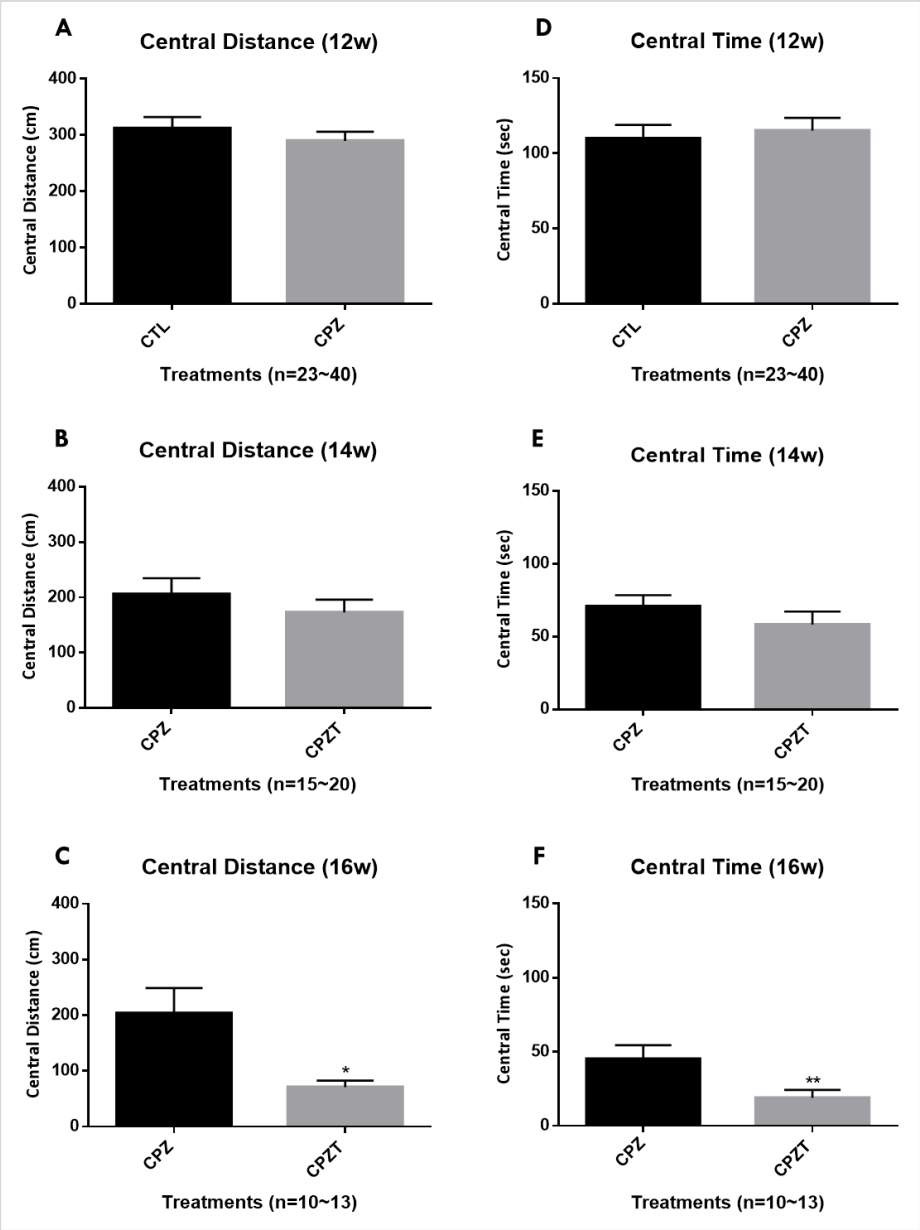

Figure 4.2: The effect of LFMS on anxiety-like behaviour. (A) The central time and central distance in the control and demyelinated mice at the end of twelve weeks of CPZ feeding (n=23~40 per group). (B) The central distance in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal (n=15~20 per group). (C) The central distance in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal (n=10~13 per group). (D) The central time in the control and demyelinated mice at the end of twelve weeks of CPZ feeding (n=23~40 per group). (B, E) The central time in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal (n=15~20 per group). (F) The central time in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal (n=10~13 per group). Data are expressed as means ± SEM. *P<0.05, **P<0.01 vs. CPZ.

4.1.3 Chronic CPZ intake resulted in severe cognitive impairment, while LFMS treatment significantly improved cognitive function

Working Memory: Y-maze was implemented to assess the effects of the CPZ and LFMS on cognitive function. The percentage of spontaneous alternations was evaluated as a measure of working memory. Unpaired t-tests results showed a significant reduction in the percentage of the spontaneous alternations of the CPZ mice compared to CTL mice (*P<0.05; Figure 4.3A). The CPZT mice showed a higher percentage of the spontaneous alternations compared to the CPZ mice after LFMS treatment for two weeks (14w) (*P<0.05; Figure 4.3B). At 16w, there was no significant difference observed in both the groups of mice, suggesting that the longer duration of the treatment does not affect the distance or the mice get accustomed to the maze, hence showing no significant difference between the groups. Thus, Y-maze results demonstrated a significant reduction in the percentage of spontaneous alternations after chronic CPZ intake, which was found to be significantly improved by LFMS treatment but only at 14 weeks (Figure 4.3). We did not notice the significant being translated to 16 weeks which could be possible due to the repeated exposure that led to the mice being aware of the maze pattern.

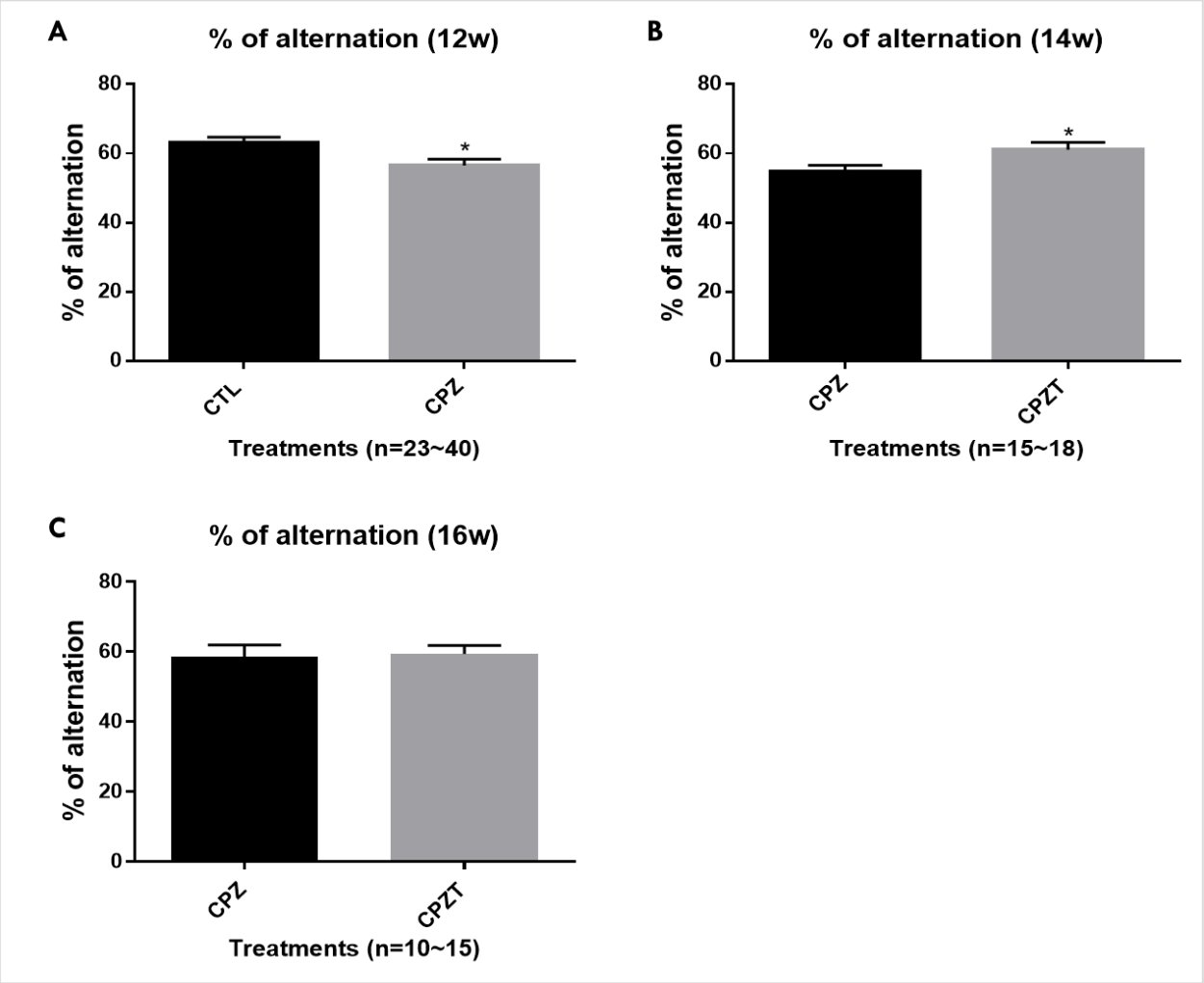

Figure 4.3: The effect of LFMS on Cuprizone-induced working memory deficits. (A) The percentage correct spontaneous alternations in the control and demyelinated mice at the end of twelve weeks of CPZ feeding (n=23~40 per group). (B) The percentage correct spontaneous alternations in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal (n=15~18 per group). (C) The percentage correct spontaneous alternations in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal (n=10~15 per group). Data are expressed as means ± SEM. *P<0.05 vs. CPZ.

4.1.4 Chronic CPZ intake leads to a high-level depressive state

Depression-like Behaviour: Tail Suspension Test (TST) was used to evaluate the effects of the CPZ and LFMS on depression-like behaviour. The immobility in the last four minutes of the test

period was measured as depression-like behaviour. Unpaired t-tests indicated a significant increase in the time immobile of the CPZ mice compared to the CTL mice at the end of twelve weeks CPZ intake (*P<0.05; Figure 4.4 A). The CPZT mice showed a gradual trend of reduction in the time immobile compared to the CPZ mice after LFMS treatment at both 14w and 16w (P>0.05; Figure 4.4 B, C). Thus, TST results suggest a significant increase in the immobile time with chronic CPZ exposure, and the immobility gradually reduced after LFMS treatment but did not significantly change (Figure 4.4).

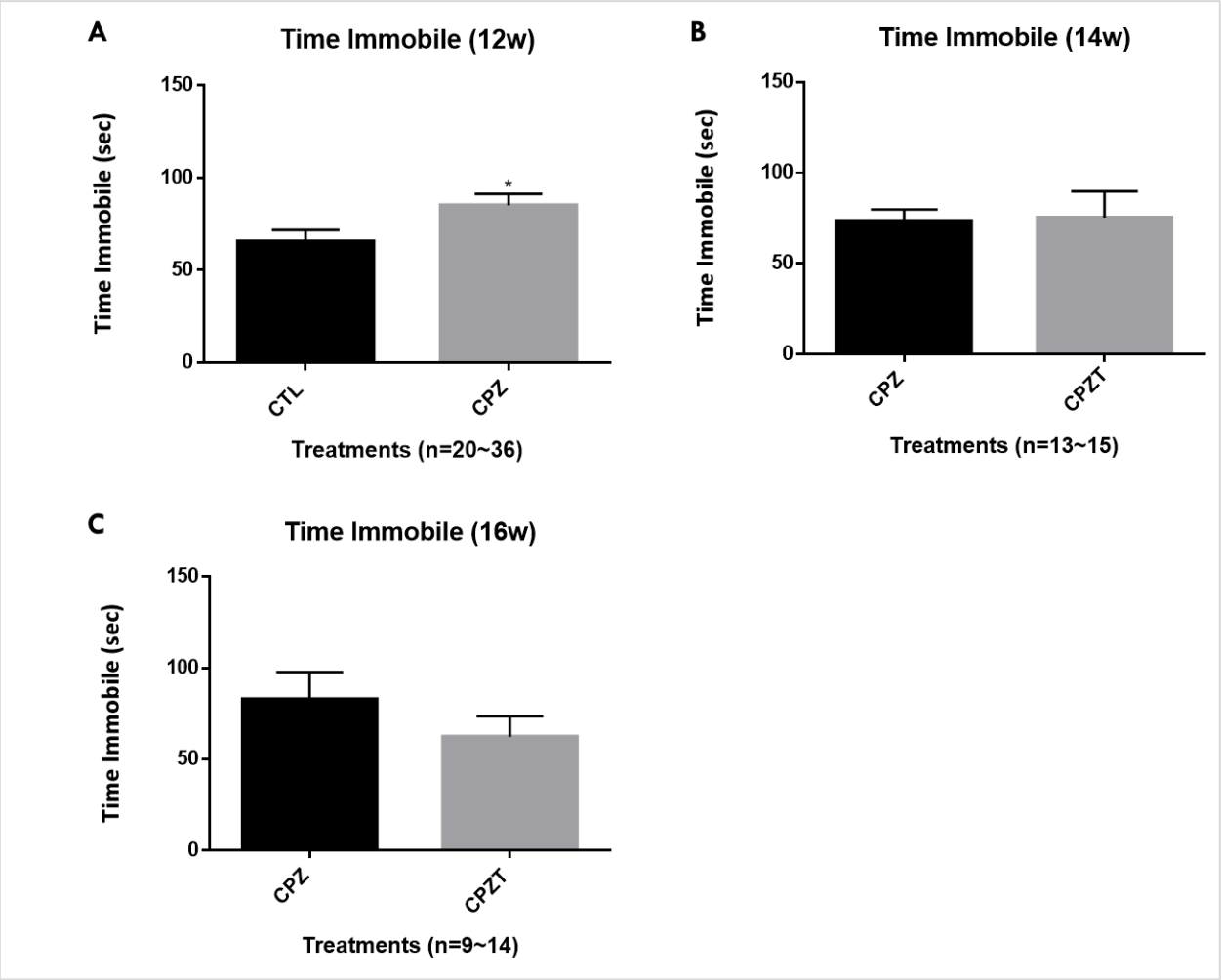

Figure 4.4: The effect of LFMS on Cuprizone-induced depression-like behaviour. (A) The time immobile in the control and demyelinated mice at the end of twelve weeks of CPZ feeding (n=20~36 per group). (B) The time immobile in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal (n=15~18 per group). (C) The time immobile in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal (n=10~15 per group). Data are expressed as means ± SEM. *P<0.05 vs. CPZ.

4.1.5 Chronic CPZ intake significantly reduced mice weight, along with significant weight regain after LFMS

Mice Weight: Weight loss is used as a clinical indicator to evaluate the CPZ-induced demyelination severity. Mice were weighed twice a week. Unpaired t-tests evaluated the severity of demyelination induced by CPZ exposure, possibly leading to a reduction in the weight. The mean body weights of the mice fed with 0.2% CPZ for 12 weeks were significantly less than that of the CTL group mice (****P<0.0001; Figure 4.5A). The CPZT mice showed a higher body weight than the CPZ mice when treated with LFMS for two weeks (14w) (*P<0.05; Figure 4.5 B). After LFMS treatment for four weeks (16w), the CPZT mice showed a significant increase in body weight than CPZ fed mice (****P<0.0001; Figure 4.5 C). Thus, a significant weight reduction was observed with chronic CPZ diet and regained after LFMS treatment, which was statistically significant, as shown in Figures 4.5 B and C.

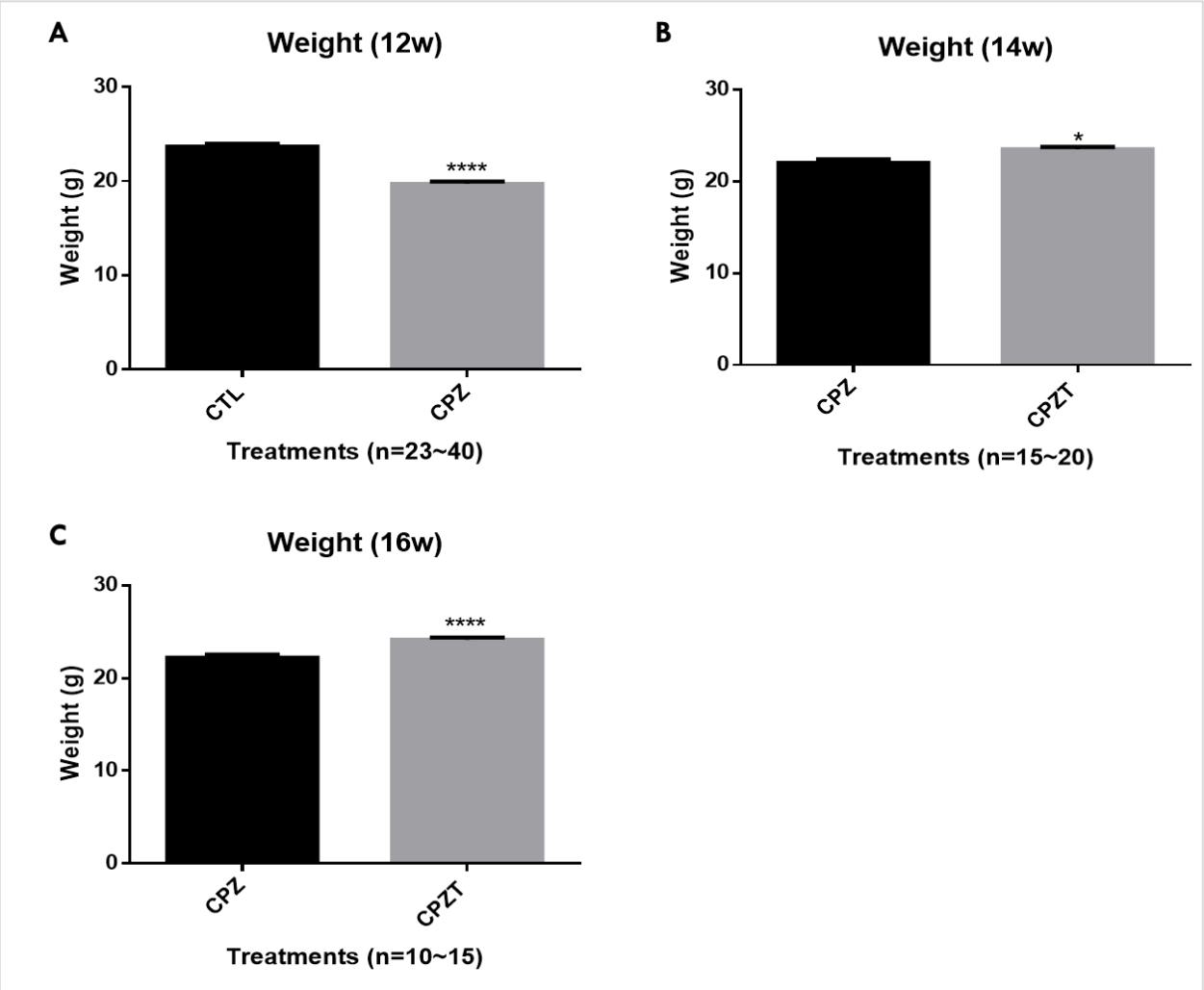

Figure 4.5: The effect of LFMS on Cuprizone-induced weight loss. (A) The weight in the control and demyelinated mice at the end of twelve weeks of CPZ feeding (n=23-40 per group). (B) The weight in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal (n=15-20 per group). (C) The weight in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal (n=10-15 per group). Data are expressed as means ± SEM. *P<0.05, ****P<0.0001 vs. CPZ.

4.2 Biochemical and pathological tests

4.2.1 LFMS treatment enhances the expression of MBP and MOG

Myelin basic protein (MBP) and Myelin oligodendrocyte glycoprotein (MOG) were used to assess the myelin content and myelin formation associated protein, respectively. MBP indicates the myelin restoration and MOG suggests the maturation of myelinated oligodendrocyte. Western blots were performed using antibodies directed against MBP or MOG. The samples used for the Western blotting were obtained from the prefrontal cortex (PFC) of mice, and β-actin was used as a loading control.

There was a significant decrease in the MBP expression in the 12w CPZ mice when compared to the ones with the regular diet (**P<0.01; Figure 4.6A, B). At 14w, the LFMS-treated mice (CPZT) showed a significant increase in the MBP expression compared to the CPZ mice (CPZ) (*P<0.05; Figure 4.6A, C). When treated for another two weeks (16w) with LFMS, a significant increase in the MBP expression was observed in the CPZT mice compared to the CPZ group (*P<0.05; Figure 4.6A, D). Thus, chronic CPZ exposure significantly reduced the MBP expression in the PFC. After the LFMS treatment, there was an increased MBP expression. (Figure 4.6).

When treated with twelve weeks of CPZ diet, the CPZ mice showed a significant decrease in the MOG expression compared to the mice with regular diet (**P<0.01; Figure 4.7A, B). After LFMS treatment for two weeks (14w), the CPZT mice showed a significant increase in the MOG expression compared to the CPZ mice (*P<0.05; Figure 4.7A, C). After four weeks of LFMS treatment (16w), a significant increase in the MOG expression was demonstrated in the CPZT mice than the CPZ mice (*P<0.05; Figure 4.7A, D). Thus, chronic CPZ exposure significantly decreased the

MOG expression in the PFC, which was significantly increased after LFMS treatment at both 14w and 16w (Figure 4.7).

In summary, chronic CPZ exposure significantly reduced both the MBP and MOG expressions in the PFC, which were significantly increased after LFMS treatment at both 14w and 16w.

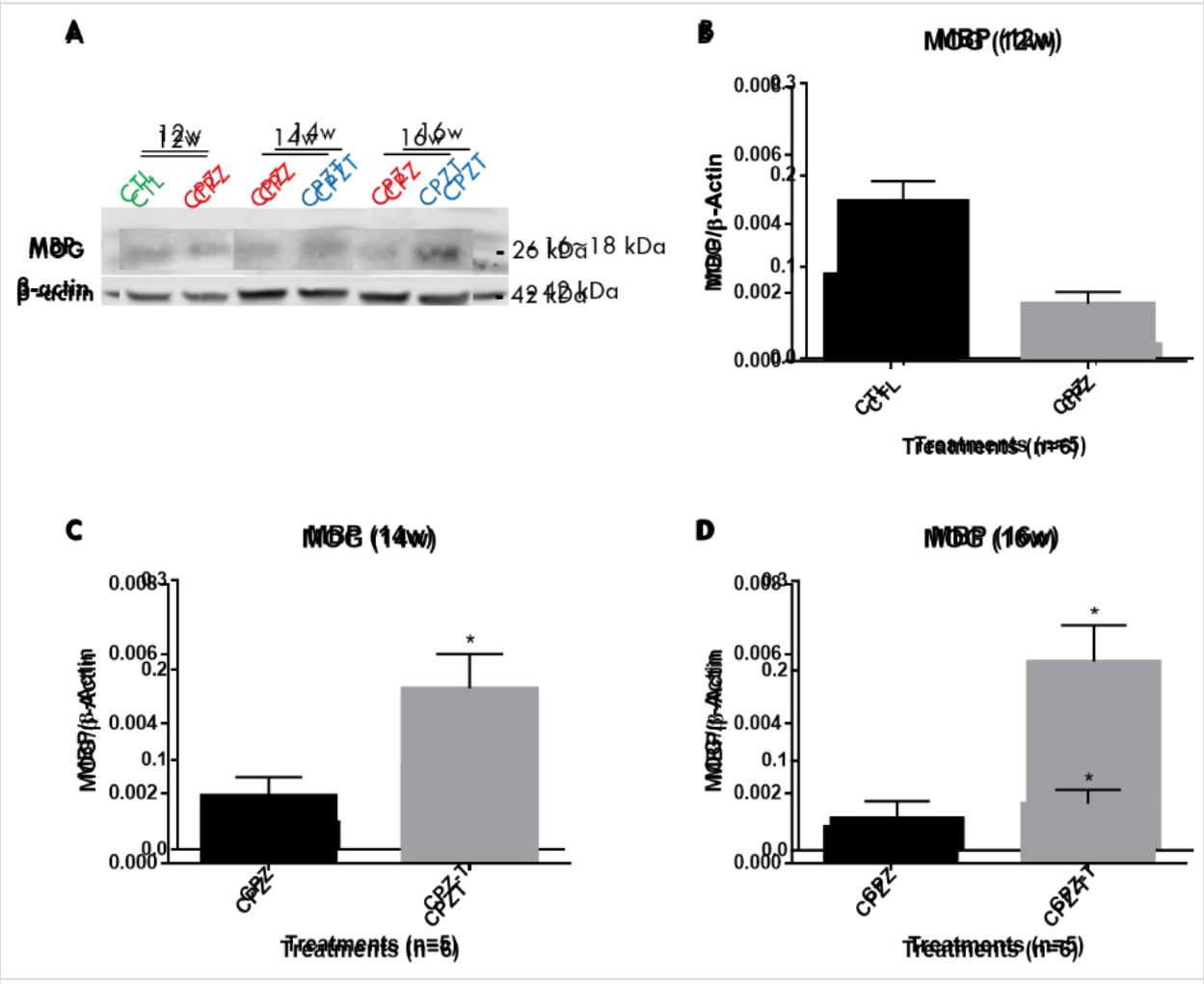

Figure 4.6: The effect of LFMS on myelin oligodendrocyte (MBP) expression (MOG) expression in the prefrontal cortex. (A) The expression of MBP was detected by Western Blot and was used as a loading control for the PFC. (B) The relative MBP/β-actin ratio in the control and the LFMS-treated rats at the end of twelve weeks of CPZ feeding. (C) MBP/β-actin ratio in the control and the LFMS-treated rats after two weeks of removal of CPZ from the diet and addition of normal drinking water. Results are expressed as mean ± SEM (n=5 per group). *P<0.05 vs. CPZ.

Figure 4.6: The effect of LFMS on myelin oligodendrocyte (MBP) expression (MOG) expression in the prefrontal cortex. (A) The expression of MBP was detected by Western Blot and was used as a loading control for the PFC. (B) The relative MBP/β-actin ratio in the control and the LFMS-treated rats at the end of twelve weeks of CPZ feeding. (C) MBP/β-actin ratio in the control and the LFMS-treated rats after two weeks of removal of CPZ from the diet and addition of normal drinking water. Results are expressed as mean ± SEM (n=5 per group). *P<0.05 vs. CPZ.

4.2.2 LFMS treatment lowered the expression of GFAP and TNFα, which are enhanced in CPZ-treated mice

Astrogliosis and Microglia Activation: Western blots were performed to assess inflammation using Glial Fibrillary Acidic Protein (GFAP) (was used to assess Astrogliosis) and Tumor Necrotizing Factor-alpha (TNFα) (a general marker of Microgliosis). Western blots were performed using antibodies against GFAP or TNFα. Unpaired t-tests were performed to evaluate the results. All loading samples were obtained from the prefrontal cortex (PFC) and β-actin used as a loading control. The 12w CPZ mice demonstrated significantly higher expression of the GFAP compared to the mice with regular diet (**P<0.01; Figure 4.8A, B). No significant difference in the GFAP expression was observed between the CPZT mice and the CPZ mice after two weeks of LFMS treatment (14w) (P>0.05; Figure 4.8A, C). After LFMS treatment for another two weeks (16w), the CPZT mice showed a trend of reduction in the GFAP expression when compared to the CPZ mice (*P<0.05; Figure 4.8A, D). Thus, chronic CPZ exposure significantly increased the GFAP expression in the PFC, while LFMS treatment showed a decreasing trend in the GFAP expression after four weeks of treatment (16w) (Figure 4.8).

The 12w CPZ mice demonstrated a significantly enhanced the expression of TNFα compared to the regular diet (**P<0.01; Figure 4.9A, B). After two weeks of LFMS treatment (14w), the CPZT mice showed a reduction trend in the TNFα expression when compared to the CPZ mice (P>0.05; Figure 4.9A, C). A significant reduction in the TNFα expression in the CPZT mice compared to the CPZ mice was observed after LFMS treatment for four weeks (16w) (*P<0.05; Figure 4.9A, D). Thus, chronic CPZ exposure significantly increased the TNFα expression in the PFC, which was significantly reduced after four weeks of LFMS treatment (Figure 4.9).

In summary, chronic CPZ exposure significantly increased the GFAP and the TNFα expression in the PFC; after the LFMS treatment, there was a significantly reduced TNFα expression and a trend of reduction in the GFAP expression at 16w.

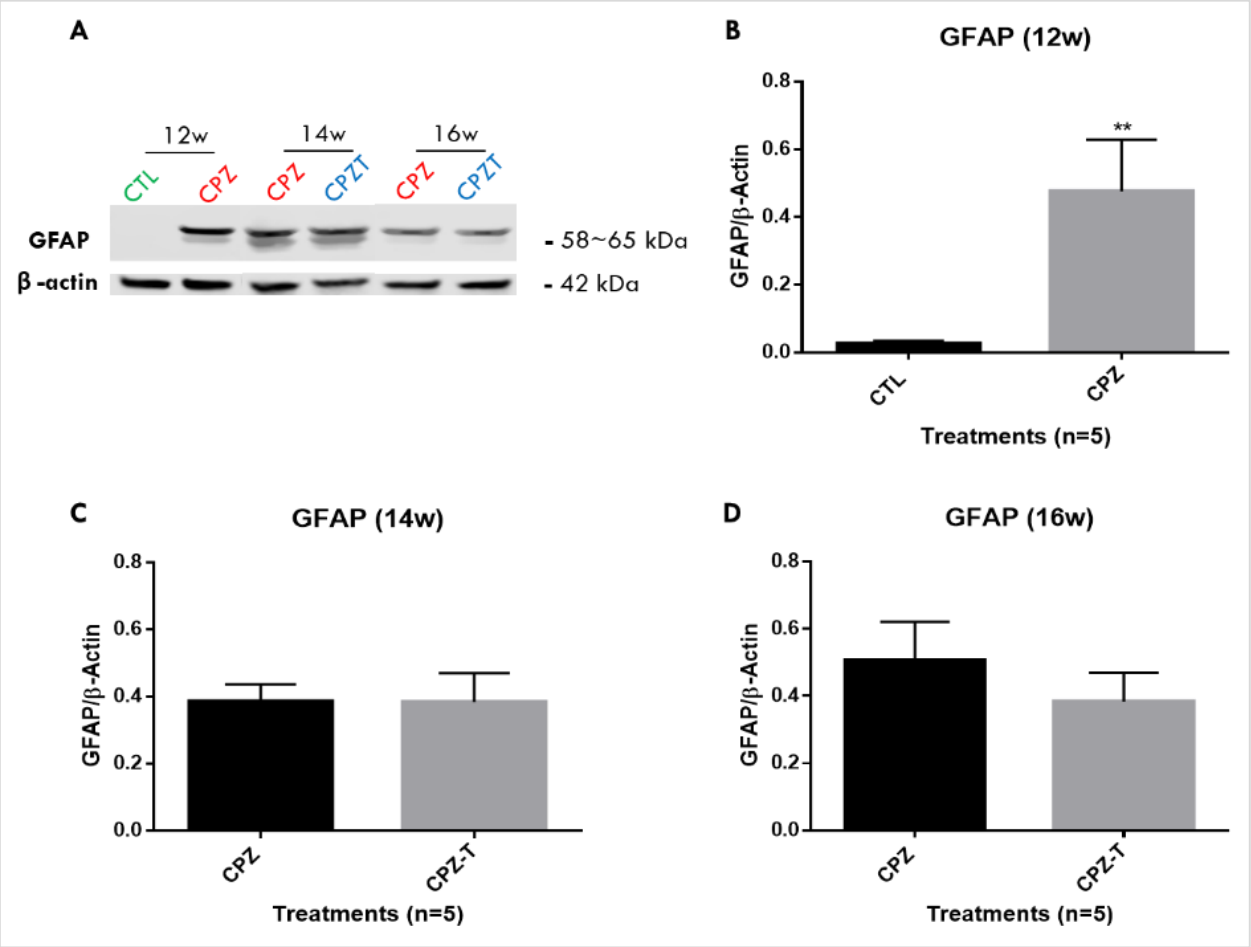

Figure 4.8: The effect of LFMS on glial fibrillary acidic protein (GFAP) expression in the prefrontal cortex (PFC). (A) The expression level of GFAP was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative GFAP/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative GFAP/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative GFAP/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). **P<0.01 vs. CPZ.

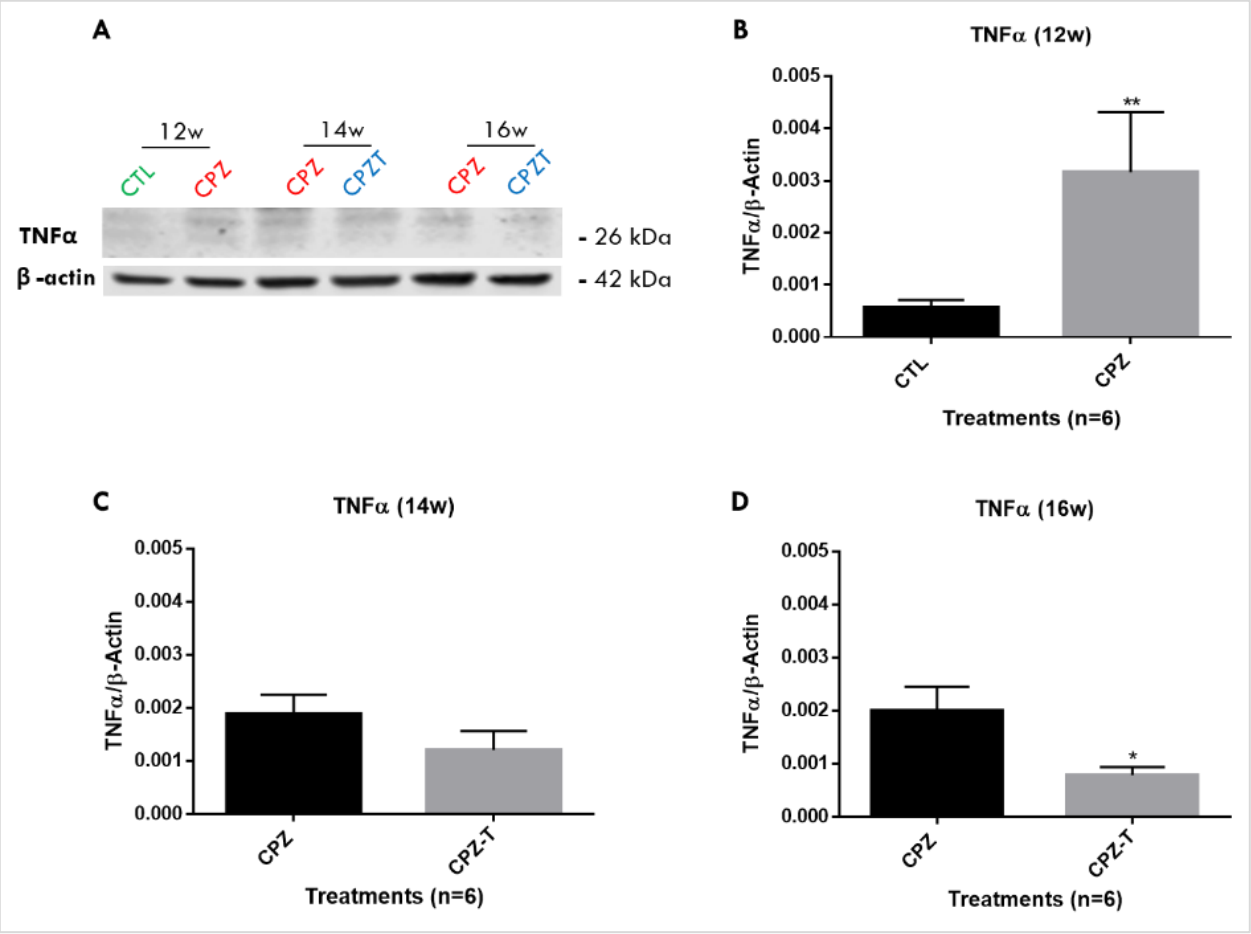

Figure 4.9: The effect of LFMS on tumour necrosis factor-alpha (TNFα) expression in the prefrontal cortex (PFC). (A) The expression level of TNFα was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative TNFα/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative TNFα/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative TNFα/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 vs. CPZ.

4.2.3 LFMS treatment decreased the Olig-2 expression and increased the GST-π expression

Oligodendrocyte transcription factor 2 (Olig-2) and Glutathione S-transferase pi (GST-π) were used to measure the amount of OL lineage cells and mature OLs, respectively. Western blots were performed using both the antibodies. All loading samples were homogenized from the prefrontal cortex (PFC) and β-actin used as a loading control.

A significant increase in Olig-2 expression in the CPZ mice was observed when compared to the 12w CTL mice (*P<0.05; Figure 4.10A, B). After LFMS treatment for two weeks (14w), the CPZT mice showed a significant decrease in the Olig-2 expression compared to the CPZ mice (*P<0.05; Figure 4.10A, C). After LFMS treatment for four weeks (16w), a significant decrease in the Olig-2 expression in the CPZT mice as compared to the CPZ mice was observed (*P<0.05; Figure 4.10A, C). Thus, chronic CPZ exposure significantly increased the Olig-2 expression in the PFC. LFMS treatment decreased the Olig-2 expression at both 14w and 16w (Figure 4.10).

When treated with CPZ diet for twelve weeks, a trend of reduction in the expression of GST-π was observed when compared to the regular diet (P>0.05; Figure 4.11A, B). No significant difference in the GST-π expression was shown between the CPZT mice and the CPZ mice at 14w (P>0.05; Figure 4.11A, C). When treated with LFMS for another two weeks (16w), the CPZT mice showed a significant increase in the GST-π expression compared to the CPZ mice (*P<0.05; Figure 4.11A, D). Thus, with chronic CPZ exposure, there was a reduction trend in the expression of GST-π in the PFC, which was increased after four weeks (16w) of LFMS treatment (Figure 4.11).

In summary, chronic CPZ exposure significantly increased the Olig-2 expression in the PFC, which was significantly reduced after LFMS treatment at both 14w and 16w. Conversely, chronic CPZ exposure significantly decreased the GST-π expression in the PFC, which was significantly increased after four weeks (16w) of LFMS treatment.

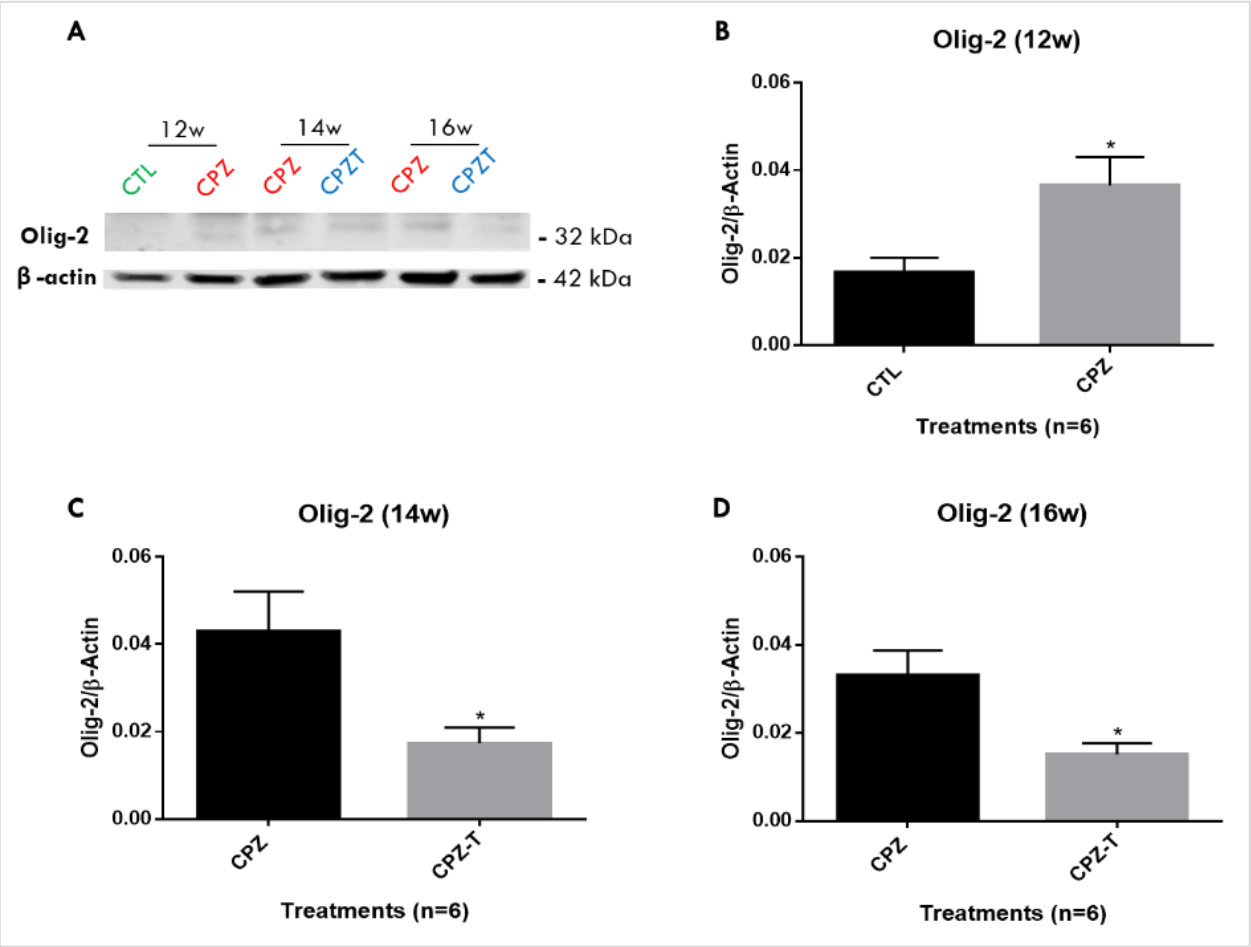

Figure 4.10: The effect of LFMS on oligodendrocyte transcription factor 2 (Olig-2) expression in the prefrontal cortex (PFC). (A) The expression level of Olig-2 was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative Olig-2/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative Olig-2/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative Olig-2/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 vs. CPZ.

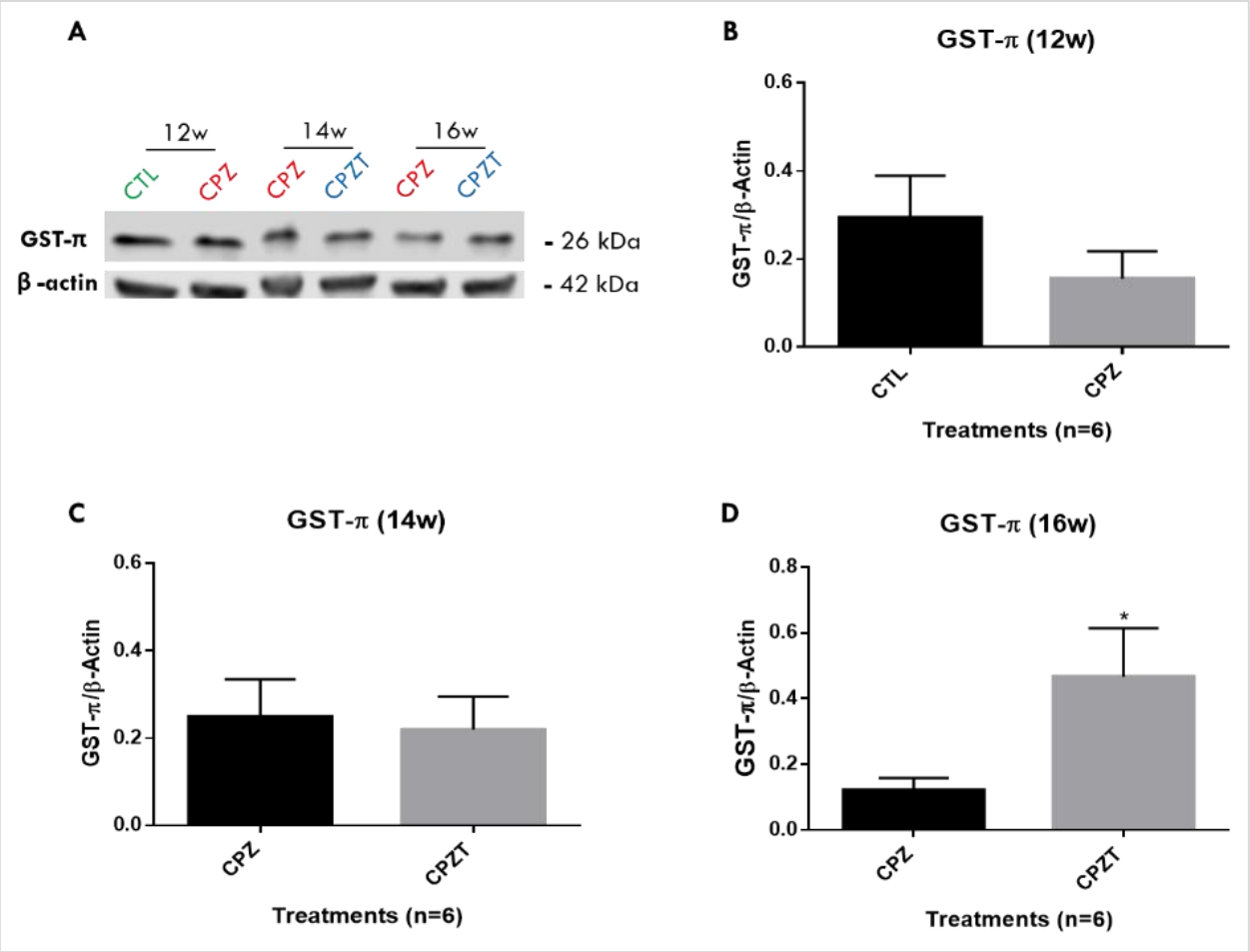

Figure 4.11: The effect of LFMS on glutathione S-transferase pi (GST-π) expression in the pre-frontal cortex (PFC). (A) The expression level of GST-π was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative GST-π/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative GST-π/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative GST-π/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 vs. CPZ.

4.2.4 Chronic CPZ intake decreased the expression of TGF-β and TGF-β-R1, and LFMS significantly enhanced TGF-β, TGF-β-R1, and TGF-β-R2

TGFβ Signaling Pathway: Transforming Growth Factor beta 1 (TGF-β1), Transforming Growth Factor beta receptor 1 (TGF-β-R1), and Transforming Growth Factor beta receptor 2 (TGF-β-R2) were immunoblotted. Western blots were performed using antibodies specific for TGF-β1, TGF-β-R1 or TGF-β-R2. Unpaired t-tests were performed to assess the statistical significance. All loading samples homogenized from the prefrontal cortex (PFC) and β-actin used as a loading control. A significant reduction in the TGF-β1 expression at 12w was observed in the CPZ mice when compared to the CTL mice (*P<0.05; Figure 4.12A, B). After LFMS treatment for two weeks (14w), the CPZT mice showed an increasing trend in the TGF-β1 expression when compared to the 14w CPZ mice (P>0.05; Figure 4.12A, C). After LFMS treatment for another two weeks (16w), the CPZT mice showed a significant increase in the TGF-β1 expression compared to the CPZ mice (*P<0.05; Figure 4.12A, D). Thus, chronic CPZ exposure significantly decreased the TGF-β1 expression in the PFC, which was significantly increased after four weeks of LFMS treatment (16w) (Figure 4.12).

After twelve weeks of CPZ feeding, the CPZ fed mice showed a significant reduction in the TGF-β-R1 expression when compared to the CTL mice (*P<0.05; Figure 4.13A, B). The 14w CPZT mice showed increased the TGF-β-R1 expression compared to the CPZ mice after LFMS treatment for two weeks (14w) (**P<0.01; Figure 4.13A, C). After LFMS treatment for another two weeks (16w), the CPZT mice showed a significant increase in the TGF-β-R1 expression compared to the CPZ mice (*P<0.05; Figure 4.13A, D). Thus, chronic CPZ exposure significantly reduced the TGF-β-R1 expression in the PFC, while LFMS treatment significantly increased the TGF-β-R1 expression, especially after two weeks of treatment (14w) (Figure 4.13).

A significant reduction in the TGF-β-R2 expression in the 12w CPZ mice was observed when compared to the 12w CTL mice ($P>0.05$; Figure 4.14A, B). After two weeks of LFMS treatment (14w), the CPZT mice showed a significant increase in the TGF-β-R2 expression compared to the CPZ mice ($*P<0.05$; Figure 4.14A, C). A significant increase in the TGF-β-R2 expression in the CPZT mice compared to the CPZ mice was observed after four weeks of LFMS treatment implemented (16w) ($*P<0.05$; Figure 4.14A, D). Thus, after chronic CPZ exposure, there was a decreased trend in the expression of TGF-β-R2 in the PFC, which was significantly increased after LFMS treatment (Figure 4.14).

In summary, after chronic CPZ exposure, there was a significant reduction in the TGF-β1 and the TGF-β-R1expression and a reducing trend in the TGF-β-R2 expression at 12w. After LFMS treatment, there was a significant increase in the TGF-β1, TGF-β-R1, and TGF-β-R2 expression.

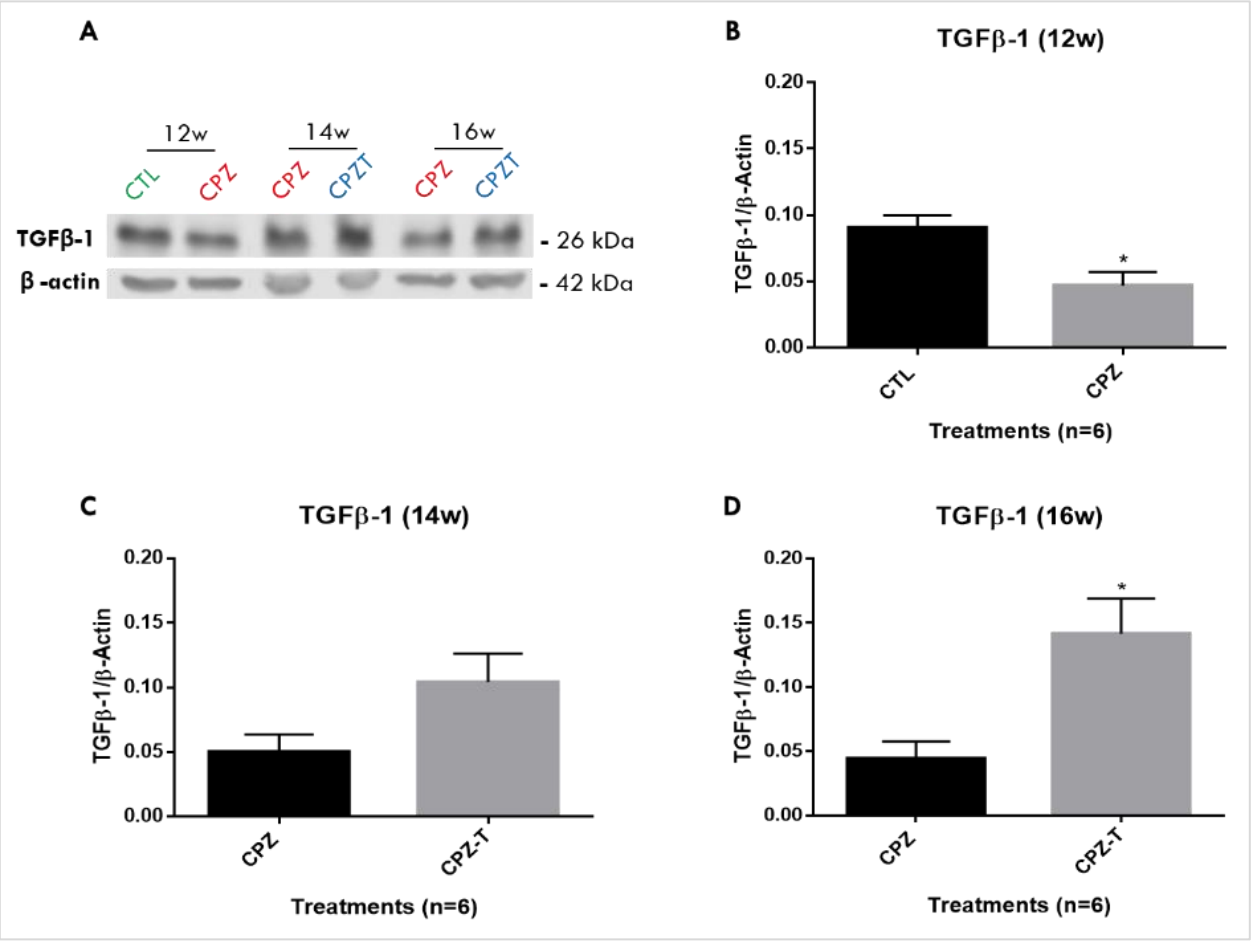

Figure 4.12: The effect of LFMS on transforming growth factor-beta 1 (TGF-β1) expression in the prefrontal cortex (PFC). (A) The expression level of TGF-β1 was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative TGF-β1/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative TGF-β1/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative TGF-β1/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 vs. CPZ.

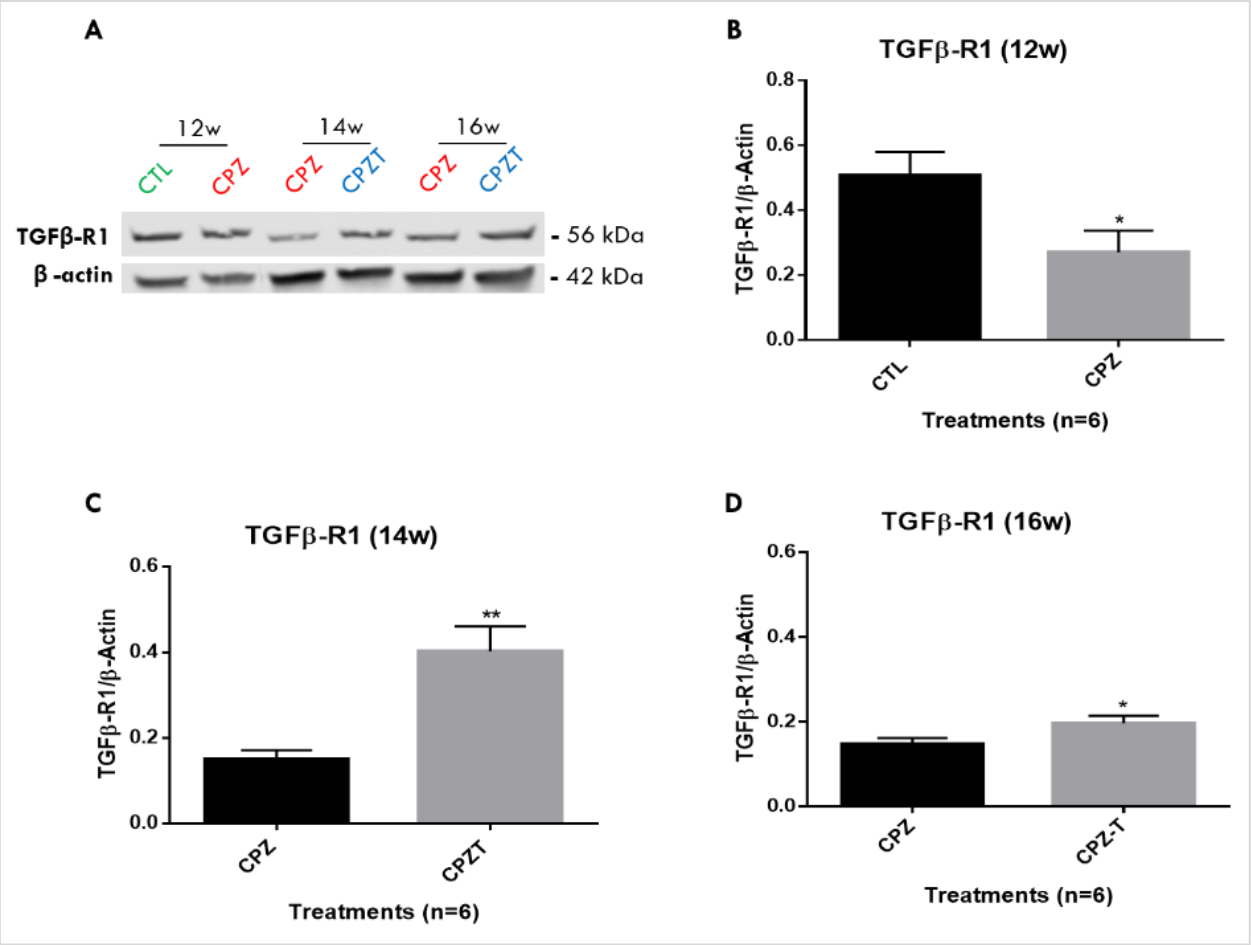

Figure 4.13: The effect of LFMS on transforming growth factor-beta receptor 1 (TGF-β-R1) expression in the prefrontal cortex (PFC). (A) The expression level of TGF-β-R1 was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative TGF-β-R1/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative TGF-β-R1/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative TGF-β-R1/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 vs. CPZ.

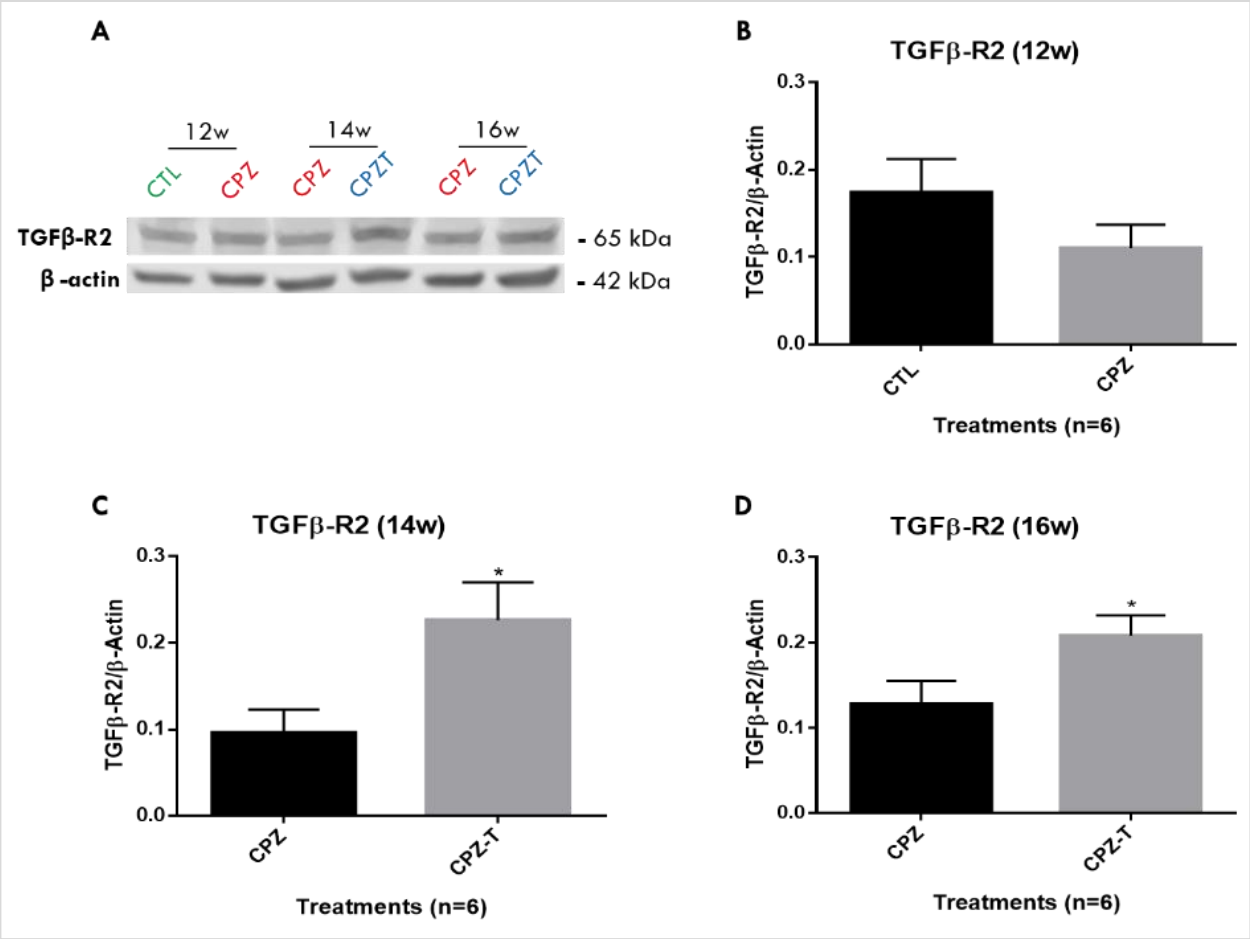

Figure 4.14: The effect of LFMS on transforming growth factor-beta receptor 2 (TGF-β-R2) expression in the prefrontal cortex (PFC). (A) The expression level of TGF-β-R2 was detected by Western Blot. β-Actin was used as a loading control, and samples are from PFC. (B) The relative TGF-β-R2/β-actin ratio in the control and demyelinated mice at the end of twelve weeks of CPZ feeding. (C) The relative TGF-β-R2/β-actin ratio in the Sham and LFMS treated mice after two weeks of remyelination and CPZ withdrawal. (D) The relative TGF-β-R2/β-actin ratio in the Sham and LFMS treated mice after four weeks of remyelination and CPZ withdrawal. Results are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 vs. CPZ.

5 DISCUSSION

Low Field Magnetic Stimulation (LFMS) is a non-invasive neurostimulation device that improves cognitive deficits after acute demyelination [111, 148]. LFMS also has neuroprotective and anti-inflammatory effects in the CNS [111, 148, 175, 176]. Moreover, a few clinical studies reported the beneficial effects of LFMS in mood disorders and cognitive-impaired neuropsychiatric disorders [140, 141, 143-145]. Nevertheless, it is difficult to determine whether the beneficial effects of LFMS implementation are due to the promotion of remyelination or because of the protection from demyelination. Thus, the CPZ-induced demyelination mouse model was utilized to investigate cognitive impairments and mood disturbances associated with chronic demyelination, neuroinflammatory response, and OL loss.

In the present study, we observed that CPZ feeding resulted in demyelination-related cognitive deficits and depression-like behaviour in twelve weeks, indicating that CPZ-induced chronic demyelination model was successfully established at the time point for LFMS treatment and behavioural tests. After LFMS treatment for two weeks and four weeks, the following behavioural changes were observed: increased awareness in the face of danger, improved cognitive function, lower level of the depressive state, and normalization of the body weight.

Notably, the unimpaired locomotor function was observed in all groups after OFT. This test was used as a foundation in our study to confirm anxiety-like behaviour and cognitive function. Consistent with previous researches on acute demyelination, LFMS treatment also demonstrated an accompanied increase in awareness in the face of danger and improved working memory deficits in mice with chronic exposure to CPZ (dose of 0.2%) [143-145, 148]. It is well known that chronic CPZ exposure imposes significant stress on the testing mice, but LFMS application produced a

reducing trend in the depressive state, suggesting the antidepressant effects of LFMS treatment [140, 141, 148].

Consistent with the results from the behavioural tests, we also demonstrated that chronic CPZ feeding resulted in biochemical and pathological changes, namely chronic demyelination, astrogliosis, microglial activation, mature OL loss, and TGF-β signalling pathway suppression. After LFMS treatment for two weeks and four weeks, we observed the following changes based on the biochemical tests: a recovery of myelin sheath integrity, amelioration of astrogliosis and microgliosis, promotion of a marker of OL differentiation, and enhancement of TGF-β receptors.

At twelve weeks, there was an evident loss of myelin in chronic CPZ-fed animals, which in turn was associated with dramatic down-regulation of the MBP expression, which has been previously recorded [153, 158]. A significant increase in the MBP expression after LFMS treatment supported a myelin restoration. To better understand the specific mechanisms of remyelination, further investigation is required. We propose the use of markers specific to the Node of Ranvier, to establish remyelination and also corroborate the location of restored myelin. The results of MOG showed a similar trend as MBP, which could be explained by the quantitative and functional differences between MBP and MOG. MBP is the major component of CNS myelin and plays an essential role in myelin compaction when establishing myelin sheath [60, 63]. However, MOG only constitutes 0.01-0.05% of the CNS myelin protein and is specifically expressed at the outer surface of the myelin sheath and OL plasma membrane [177-179]. The function of MOG remains unclear, but previous studies have suggested that MOG completes and maintain myelin sheath integrity, and serves as an adhesive molecule in cell-cell interaction [180, 181]. MOG also plays a more prominent role in regulation and communication after the myelin sheath establishment. This function is

specific to MOG. Therefore, MOG served as a regulator rather than components, thus showing a relatively lower quantitative requirement compared to MBP expression.

Chronic administration of CPZ also triggers astrogliosis and microglial activation [157]. Microglia are highly dynamic cells characterized by phenotypical and functional polarization (M1 or M2) [90]. It is suggested that activated M1 microglia are involved in neurotoxicity and pro-inflammation [93, 94]. Several M1 markers have been studied in MS, including tumour necrosis factor (TNF)-α, interleukin (IL)-1 β, IL-6, and nitric oxide (NO) throughout the active, destructive demyelinated lesion [95-97]. Our study investigated microglia activation in response to inflammatory attacks and the polarization to M1 by using TNFα as a representative marker. The significantly enhanced expression of TNFα after CPZ exposure for twelve weeks might indicated microglia activation after inflammation [182]. To identify more than one protein double immunostaining will be utilized. To identify the source of TNFα we can use it along with M1 markers. After LFMS treatment for four weeks, the significant reduction of TNFα expression suggested a restrained polarization to M1 and an amelioration of inflammatory responses [91]. The indication of TNFα was further supported by the promotive M2 polarization, which was observed by TGF-β expression in the study. Based on the results above, inhibition of M1 microglia activation could provide potential protection of the Grey Matter (GM) and White Matter (WM) in the MS mouse model. Microglia polarization could be used in designing therapeutic targets [183, 184]. These results can be further confirmed by using other marker of microglia, like Ionized calcium-binding adaptor protein (Iba)-1, interleukin-1 (IL)-1 β, IL-6 and nitric oxide (NO) [95-97]. This is so because TNF-apla is not only secreted by microglia, and it could also come from activated astrocytes (A1 astrocytes as discussed below), infiltrating macrophages, etc [185-188].

A similar trend was observed in the results of GFAP. Consistent with previous studies, there was a significant increase in the GFAP expression in CPZ-fed animals, indicating strong astrogliosis occurred as a response to chronic CPZ feeding [157, 189, 190]. Supported by various studies on GFAP-positive astrocytes in disorders, it is known that astrocyte might possess a dual role, which could be either protective or detrimental in the case of MS [191-194]. A recent study showed that activated microglia caused neurotoxic reactive astrocytes (also termed as A1) by secreting interleukin-1β (IL-1β) and TNFα [182, 185]. A1 astrocytes lose the ability to promote neuronal survival, outgrowth, and induce the death of neurons and OLs [185]. Therefore, we introduced GFAP as another marker that indicates an inflammatory response.

Interestingly, our study also presented an unsynchronized amelioration on astrogliosis and microglia activation. After four weeks of LFMS treatment, there was a decreasing trend in the GFAP expression, which was not statistically significant as TNFα. These changes indicate that astrogliosis persisted for weeks in remyelination, which is in contrast to the quick amelioration of microglia activation in the MS tissue samples [195, 196].

The OL lineage is composed of OPCs and mature OL, which play a crucial role in remyelination [197]. As described in previous studies, OPCs proliferate and migrate to demyelinated areas in MS [75, 78]. Remyelination is initiated from the directional differentiation of OPCs into pre-myelinating OLs [76, 77]. The pre-myelinating OLs wrap around demyelinated axons, thus forming mature OLs supporting new myelin reconstruction [76, 77]. In the present study, mature OLs marked with GST-π were extensively damaged, possibly due to the oxidative stress and mitochondrial dysfunction generated by the excitotoxicity of chronic CPZ intake [70-72]. Promotion in OL differentiation and maturation had been identified with enhanced the GST-π expression when treated with LFMS for four weeks. The reduction of free radicals was controlled by the parallel increase in the

expression of GST- π. However, a reverse trend was seen in immunoblots with Olig-2. It is widely accepted that Olig-2 is a robust and representative marker for OL lineage cells [198-200]. Notably, prior studies have demonstrated that the expression of Olig2 is transient in immature astrocytes and downregulated progressively in mature astrocytes [201, 202]. Therefore, we introduced Olig-2 in this study as an indirect marker for OPCs when combined with the results of GST-π. A reduction trend in the GST-π expression after twelve weeks of CPZ exposure, along with the significant increase in the Olig-2 expression, suggested an increase in the proliferation of OPCs. After LFMS treatment for four weeks, the considerable reduction in Olig-2 expression suggested an amelioration of OPCs differentiation. This is further indicative of the enhancement of OL differentiation and maturation.

In the present study, the involvement of the TGF-β pathway in MS was studied in the process to understand better the molecular changes initiated due to LFMS leading to improved cognitive function and remyelination. In contrast with the polarization to M1, previous studies have revealed that the shifted activation of M2 microglia involves tissue repairing by cleaning myelin debris and producing anti-inflammatory cytokines, represented by IL-10, TGF-β, and glucocorticoids [92, 98, 102]. Despite this, it had been reported that TGF-β could regulate CNS myelination and a decrease in TGF-β expression was therefore expected to correlate with an attenuation of remyelination in the spinal cord after toxin-induced demyelination [121, 131]. Based on the previous studies, we also suggest that LFMS probably promotes remyelination in a chronic CPZ-induced demyelination model through the TGF-β pathway. Significantly decreased TGF-β1 and TGFβ-R1 expression at 12w supported that chronic CPZ exposure suppressed the TGF-β signalling pathway, and there was an implicit correlation between chronic demyelination and TGF-β pathway. When treated with LFMS, enhanced expression in TGF-β1, TGFβ-R1, and TGFβ-R2 was recorded. Interestingly,

when compared to the results of Y-maze at 14w and 16w, the correlative enhancement of TGFβ signalling cascade and % of alternation further suggested that LFMS promotes remyelination possibly via enhancing TGF-β signalling cascade. According to previous work, TGF-β signalling cascade also plays as a key in long term memory [203].

Notably, previously unpublished work from our lab has indicated that LFMS also improves cognitive function and is accompanied by increased TGF-β1 expression in the acute demyelination model. These findings further support the role of the TGF-β pathway in the MS mouse model. TGF-β signalling possibly led to remyelination, and improved cognition after LFMS treatment in both acute and chronic CPZ treated mice. In summary, these results suggest that the TGF-β signalling pathway could be essential for the process of remyelination. It further supports detailed studies to understand the molecular mechanism of the TGF-β signalling after LFMS treatment and the remyelination process, thus using it as a potential treatment option for MS. These results can be further confirmed after determined the cellular sources of TGF-β by using other marker related to M2, like IL-4, IL-10, and Cluster of Differentiation 163 (CD163) [98, 204]. To know the mechanism of TGF-β signal transduction better, the detection of downstream molecules are required, such as Smads and phosphorylated Smads.

In the present study, we have shown for the first time, the effects of LFMS treatment on the chronic CPZ-induced demyelination model. LFMS improved cognitive function and alleviated mood disturbances after chronic demyelination. Further, we have shown that LFMS promoted remyelination and OL maturation by ameliorating inflammatory responses. Most surprisingly, changes in MOG expression by immunoblotting were recorded in CPZ-induced demyelination for the first

time. These observations have been made previously in the experimental autoimmune encephalomyelitis (EAE) model. Finally, the TGF-β pathway was identified as a possible therapeutic target as it might be involved in the remyelination process after LFMS treatment.

To better understand the effects of LFMS treatment, we can use immunohistochemical analysis (IHC). IHCs could shed light on understanding the pathological changes in LFMS-promoted remyelination. Double staining could be introduced using GFAP with IL-10, and BrdU with GST-π. Double staining would allow us to answer a few questions, such as the location of inflammatory cytokines and the location of OPCs differentiated during the demyelination and remyelination processes. We would use direct markers to locate immature OLs in the tissue sections. To better understand the TGF-β cascade, the downstream signalling molecules should be immunoblotted to establish their roles in the remyelination process after LFMS treatment.

In summary, chronic CPZ exposure resulted in CI and led to a depressive state in the mice. CPZ also induced mature OL loss and loss of myelin sheath and increased inflammatory responses. Possible suppression of TGF-β signalling is indicated based on the receptor-ligand expression. LFMS treatment improved the cognitive function and ameliorated the mood disturbances based on the behavioural analysis. LFMS treatment also promoted remyelination and OL maturation, possibly by enhanced TGF-β signalling, and reduction in the ameliorated inflammatory responses.

6 LIMITATIONS

There are a few limitations in the current study which can be addressed. To further validate behavioral changes more tests can be incorporated, such as elevated plus maze (EPM) and novel object recognition test. These tests will detect the effects of LFMS treatment on the level of anxiety and

learning ability, specifically long-term memory. For microgliosis in WB, only TNF-α was investigated and used as a specific marker. However, TNF-α is not just secreted by microglia, and it could also come from A1 astrocytes, infiltrating macrophages, etc. To identify the microgliosis and further inflammatory response, other markers (Iba-1, IL-1, IL-6) of M1 microglia are required. For TGF-β signalling, the cellular sources of TGF-β and receptors were not determined. These limitations could be solved by implementing IHC, ELISA, or cell culture. Therefore, more typical markers for specific cells and the identification of the source of cytokines involved in the study are required in future studies before we made a further conclusion on current results.

7 CONCLUSIONS AND FUTURE DIRECTION

Our study has shown that LFMS improved cognitive function and ameliorated mood disturbances in the chronic demyelination of female C57BL/6 mice. The TGF-β signalling pathway might be a potential therapeutic target in MS treatment. Though we need more research to confirm the results and better understand the molecular signalling, this could be a starting point. LFMS could be a novel technique for the treatment of MS since it is non-invasive and has no known side effects.

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