Effects of Low Field Magnetic Stimulation on Cognitive Impairment and Brain Pathologies in the Cuprizone Mouse Model of Demyelination

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By

Ali Mooshekhian

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ABSTRACT

Cognitive impairment (CI) is a leading cause of disability in patients with Multiple Sclerosis (MS). Although CI has catastrophic effects on patients' quality of life, there is no approved treatment for it. Low Field Magnetic Stimulation (LFMS) is a novel non-invasive brain stimulation technique that has promising benefits in improving mood and cognitive function in animal studies of traumatic brain injury, major depressive disorder and Alzheimer's disease, suggesting a transdiagnosic cognitive benefit among different neuropsychiatric disorders. In this study, we hypothesized that LFMS can alleviate demyelination-related cognitive deficits in a cuprizone (CPZ) mouse model of MS. CPZ is a copper chelator widely used to generate brain demyelination. Feeding CPZ to young adult mice for six weeks generates diffuse demyelinating lesions in both gray matter and white matter areas, predominantly in the prefrontal cortex, hippocampus and corpus callosum.

One-hundred and twenty mice were divided into four groups (30 mice/group) and received CPZ (no, yes) and LFMS (no, yes) for up to 6 weeks, respectively. Behavioral tests including open-field test (OFT), Y-maze test and forced swim test (FST) were done after 3 and 6 weeks of treatment. Half of the mice from each group were euthanized at each time point, followed by brain immunobiology and Western blots.

The study showed that CPZ treatment caused significant working memory, short term memory and learning deficits, which were prevented with LFMS co-administration. The OFT and Y-maze test did not reveal motor function impairment in demyelinated mice. LFMS treatment also decreased the severity of demyelination in both frontal cortex and hippocampus. LFMS may exert its protective effects through modulating the expression of Transforming Growth Factor Beta-1 (TGF-β1), a target for an investigational intervention under clinical trials for MS. TGF-β1 is a critical cytokine for neurogenesis and inflammation. The results suggest the therapeutic potential of LFMS for cognitive remediation in MS patients. In addition, it provides a non-invasive and affordable approach to increase TGF-β1 in the brain for neuroprotection and neurogenesis.

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Head of the Department of Psychiatry

University of Saskatchewan

Ellis Hall, 103 Hospital Drive

Saskatoon, Saskatchewan S7N 0W8 Canada

OR

Dean

College of Graduate and Postdoctoral Studies

University of Saskatchewan

116 Thorvaldson Building, 110 Science Place

Saskatoon, Saskatchewan S7N 5C9 Canada

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Ali Mooshekhian

DEDICATIONS

This little piece of work is dedicated to four people as symbol of appreciation for one single thing that they had in common: They believed in “me” when nobody else did:

Ali Asghar

Heshmat

Azadeh

Yanbo

Ali Mooshekhian.

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LIST OF ABBREVIATIONS

BBB: Blood Brain Barrier

CCAC: Canadian Council of Animal Care

CI: Cognitive Impairment

CNS: Central Nervous System

CPZ: Cuprizone

DMTS: Disease Modifying Therapies

FST: Forced Swim Test

GBO: Gamma Band Oscillation

GFAP: Glial Fibrillary Acidic Protein

GM: Gray Matter

IL: Interleukin

LFMS: Low Field Magnetic Stimulation

MS: Multiple Sclerosis

MBP: Myelin Basic Protein

NAWM: Normal Appearing White Matter

OFT: Open Field Test

OL: Oligodendrocyte

OPC: Oligodendrocyte Progenitor Cell

PFA: Paraformaldehyde

rTMS: repetitive Transcranial Magnetic Stimulation

TGF-β: Transforming Growth Factor- Beta

TNF-α: Tumor Necrotizing Factor- Alpha

UACC: University Animal Care Committee

WM: White Matt

CHAPTER 1. BACKGROUND AND LITERATURE REVIEW

Introduction

Multiple Sclerosis (MS) is the most common cause of demyelination in the central nervous system (CNS) in humans and Cognitive Impairment (CI) is one of the most debilitating primary neuropsychiatric symptoms that often co-occur with pure neurologic symptoms, affecting up to 70% of MS patients (Beck, Metz, Svenson, & Patten, 2005; Davids, Hartwig, & Gastpar, 2004; Flensner, Landtblom, Soderhamn, & Ek, 2013; Hussain & Belderbos, 2008; Poppe, Wolfson, & Zhu, 2008; Rao, Leo, Ellington, et al., 1991; Reimer, Aderhold, Lambert, & Haasen, 2006). There are no approved treatments for CI in MS. While depression is treatable, it is under-managed due to decreased tolerance of side effects in MS patients. Untreated depression worsens CI, and CI decreases treatment response of depression in MS patients, a fact that highlights the need for finding an effective and available treatment for CI in MS.

Repetitive transcranial magnetic stimulation (rTMS) is a well-known form of non-invasive brain stimulation and is widely used for managing depression. This method has also been shown to reduce anxiety, spasticity, and depressive symptoms in MS, but the effects of rTMS on CI and MS progress is inconclusive. Low-field magnetic stimulation (LFMS) is a variation of the rTMS, and works by conveying low-intensity magnetic stimulation to various cortical and subcortical areas. Clinical studies reported rapid mood-elevating effects of LFMS in depressed patients or those with bipolar disorder, and animal studies documented improvement

of CI after gamma-band (gamma-LFMS) treatment in some non-MS models (Zhang Y. et. al., 2014; Zhen J. et. al., 2017)

In the current study, we examined the effects of gamma-LFMS on CI and the potential mechanisms related to those effects.

Cognitive impairment in MS

As one of the leading causes of disability in MS, CI greatly decreases quality of life and is associated with unemployment and social isolation (Beck, Metz, Svenson, & Patten, 2005; Davids, Hartwig, & Gastpar, 2004; Flensner, Landtblom, Soderhamn, & Ek, 2013; Hussain & Belderbos, 2008; Poppe, Wolfson, & Zhu, 2008; Rao, Leo, Ellington, et al., 1991; Reimer, Aderhold, Lambert, & Haasen, 2006). The most common affected domains of cognition in MS are attention, information processing speed, and memory. Social cognition and vasomotor skills are also widely impaired in MS patients (Chalah & Ayache, 2017; Chiaravalloti & DeLuca, 2008; Cotter et al., 2016). Although CI can start at any stage of MS, it is mostly found in progressive forms of MS (Benedict et al., 2006; Rao, Leo, Bernardin, & Unverzagt, 1991). Once CI occurs, it rarely improves. The severity of CI is associated with patient's cognitive function before MS and the age at which the disease starts (Chiaravalloti & DeLuca, 2008; Ekmekci, 2017). Disease activity (e.g. MS relapse) can worsen cognitive function temporarily but is not associated with the progress of CI (Duque et al., 2008). Up to this point, there are no established treatments for CI management in MS. Disease-modifying therapies (DMTS), memory-enhancing agents, physical exercises and other options including cognitive retraining demonstrated limited and inconclusive cognitive benefits in MS (Feinstein, Freeman, & Lo, 2015; Sumowski et al., 2018).

Cognitive impairment and MS brain pathologies

The origin of CI in MS is elusive. White matter (WM) and gray matter (GM) pathologies, including neuronal and oligodendrocyte (OL) loss, myelin damage and disrupted repair, and microglial activation have shown to be involved in CI in MS (DeLuca, Yates, Beale, & Morrow, 2015; Miller & Raison, 2016; Rimkus et al., 2018). Neuronal and OL loss could result from glutamate-mediated excitotoxicity, oxidative injury or mitochondrial failure (Haider et al., 2011; D. Mahad, Ziabreva, Lassmann, & Turnbull, 2008; D. H. Mahad, Trapp, & Lassmann, 2015).

Recently, a selective loss of GABAergic parvalbumin-positive (PV+) interneurons has been documented in both animal models (Falco, Pennucci, Brambilla, & de Curtis, 2014; Lapato et al., 2017; Potter et al., 2016) and post-mortem studies (Clements, McDonough, & Freeman, 2008; Gray, Thomas, Betmouni, Scolding, & Love, 2008). PV+ interneurons are responsible for generating gamma band oscillations (GBOs, 30–120 Hz) via synchronized inhibition of large pyramidal cell clusters (Cardin et al., 2009; Sohal, Zhang, Yizhar, & Deisseroth, 2009). GBOs (especially 40 Hz) are major role players in retaining synaptic plasticity and cognition (Buzsáki & Wang, 2012). PV+ interneurons have extensive myelination compared to other types of neurons (Hendry, Houser, Jones, & Vaughn, 1983), which enables PV+ neurons to provide metabolic and trophic support to PV interneurons (Inan et al., 2016; Kann, Papageorgiou, & Draguhn, 2014). PV+ neurons also facilitate GBOs (Inan et al., 2016; Kann et al., 2014; Kim et al., 2015). Abnormal GBOs are shown to be strongly related to CI in autism, schizophrenia and Alzheimer's disease (Brambilla & Tansella, 2007; Kochunov & Hong, 2014; Wheeler & Voineskos, 2014). Entraining gamma stimulation at 40 Hz to the brain is demonstrated to have potential anti-inflammatory and neuroprotective effects in an animal model of Alzheimer's,

evidence for possible therapeutic value in using this approach to treating neurodegeneration and immunomodulation (Iaccarino et al., 2016).

Based on various studies on animals and post-mortem brain tissues, data indicates that excitotoxicity is a major contributor to demyelination, OL death, and tissue damage in MS (Gonsette, 2008). Demyelinated axons are exposed to neurotoxic insults, oxidative stress, and energy deficiency; thus, they are vulnerable to more insult. This eventually results in irreversible axonal damage (Irvine & Blakemore, 2006). Remyelination is a natural regenerative process to restore myelin sheaths on demyelinated axons (Franklin, 2002; Patani, Balaratnam, Vora, & Reynolds, 2007; Patrikios et al., 2006). However, remyelination is compromised in chronic MS lesions (Barkhof et al., 2003). Myelin debris should be cleared by microglia in order for oligodendrocyte progenitor cells (OPCs) to be recruited for remyelination (Kuhlmann et al., 2008). Reduction in myelin debris clearance and impaired remyelination are documented to be partially due to non-efficient microglial function (Lampron et al., 2015).

Cognitive impairment and microglia function in MS

Although studying the microglia is not a primary objective of the current study, it is still essential to review the function of microglia here, for two reasons. First, microglia plays a more prominent role than T-cells in the pathology of progressive MS. The same pattern is respectively observed when Cuprizone model is compared to other animal models of MS which are based upon autoimmunity. Second, we aim to study the effects of Transforming Growth Factor Beta1 (TGF- β1), which has been documented to be involved in microglia polarization. The following paragraph will briefly summarize what is currently known about microglia as their function pertains to this study.

Cortical lesions have shown a paucity of T and B lymphocyte infiltrations, which suggests a “closed” blood brain barrier (BBB) in progressive MS (Kutzelnigg et al., 2007). Microglia are thought to have an important role in immune regulation without BBB disruption. When activated, microglia phenotype changes into M1 or M2, a phenomenon called polarization (Mori et al., 2017). M1 is the neurotoxic form due to release of excessive glutamate, nitric oxide (NO) and pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1 β, IL-6 (Ceulemans et al., 2010; Domercq et al., 2007; Gregersen, Lambertsen, & Finsen, 2000). Reduction in M1 microglia were shown to protect both GM and WM in animal models of MS (Faissner et al., 2017; Heppner et al., 2005). However, M2 plays a neuroprotective and anti-inflammatory role via removing axonal and myelin debris and producing anti-inflammatory factors (Jin & Yamashita, 2016; Karamita et al., 2017; Neumann, Kotter, & Franklin, 2009; Rawji & Yong, 2013). In cases of decreased M2 microglia, remyelination has shown to be inhibited (Kotter, Li, Zhao, & Franklin, 2006; Miron et al., 2013). Moreover, M2 malfunction is shown to be related to cognitive and emotional disturbances in other neuropsychiatric diseases including Alzheimer’s disease, stroke, and major depression (Miller, 2009; Nakagawa & Chiba, 2014; O'Connor et al., 2009; Sarlus & Heneka, 2017). Recently, activated microglia has demonstrated the ability to regulate PV+ neuronal synapses; thus increasing the synchronized GBOs. This in turn increases the expression of anti-apoptotic and neurotrophic molecules and decreases cortical neuronal apoptosis after injury (Chen et al., 2014). Entraining gamma-stimulation at 40 Hz to the brain has demonstrated possible anti-inflammatory and neuroprotective effects in an animal model of Alzheimer's disease through microglia modulation (Iaccarino et al., 2016). These findings suggest that GBO entrainment can modulate microglia function and may improve cognitive functions (Burke, Kerr, Moriarty, Finn, & Roche, 2014;

Iaccarino et al., 2016; McGeer & McGeer, 2015).

Cognitive impairment in cuprizone demyelination model

CPZ is a well-known copper chelator commonly used to generate CNS demyelination (Suzuki & Kikkawa, 1969). Adding CPZ to young adult mice diet over a six week period leads to demyelination, OPC proliferation, and activation of microglia in specific areas of the brain including the prefrontal cortex, hippocampus, cerebellar peduncles, and corpus callosum (Torkildsen, Brunborg, Myhr, & Bo, 2008). CPZ can induce diffused lesions in both GM and WM areas which is similar to periventricular normal-appearing white matter (NAWM) damage (Goldberg, Clarner, Beyer, & Kipp, 2015). When CPZ is removed from the animal's diet, spontaneous and complete remyelination is observed within six weeks (G. G. K. Matsushima & P. P. Morell, 2001). However, in case of longer exposure to CPZ (≥12 week) chronic demyelination will happen , which is also accompanied by PV+ interneuron loss, cortical atrophy, incomplete remyelination and chronic microglia activation (Zendedel, Beyer, & Kipp, 2013). The pathological events observed in chronic CPZ demyelination model is quite comparable to some important aspects of CI in MS (see details in 2.2 and 2.3).

The mechanism through which CPZ-induced demyelination happens is still elusive. It has been suggested that CPZ ingestion might lead to a state of copper deficiency which could cause mitochondrial dysfunction, oxidative stress, and OL death (Hiremath et al., 1998). It is also known that CPZ triggers microglia activation leading to release of proinflammatory cytokines. The latter phenomenon is suggested to be the cause of OL and PV+ interneuron loss (G. G. K. Matsushima & P. P. Morell, 2001). Recently, it has been shown that a disruption in microglia polarization in chronic CPZ demyelination model might be the reason behind impaired

remyelination (Berghoff et al., 2017; Clarner et al., 2012). The CPZ model has previously been shown to induce CI and depressive-like symptoms without significant motor function deficits which are commonly observed in other MS models (Xiao et al., 2008; Zhang et al., 2007; Y. Zhang et al., 2008). This provides us with an opportunity to focus on the psychiatric aspect of CPZ model demyelination and possible means of treating them (Xu, Yang, McConomy, Browning, & Li, 2010). In this study, we used the acute CPZ model to study the therapeutic effects of LFMS on CI, as well as related brain pathologies and subsequently check the possible mechanisms of those findings.

Transforming Growth Factor Beta and MS

Transforming Growth Factor Beta1 (TGF- β1) is a cytokine which has been shown to be helpful in remyelination and lowering inflammation in MS (Xie et al., 2018). This has specifically been studied in progressive MS (Nataf, Barritault, & Pays, 2017). The role of TGF- β1 in elevating the immunomodulatory ability of neural stem cells has also been recently studied (Xie et al., 2018). The exact action mechanism of TGF- β1 is still elusive. However, so far we know that TGF- β1 is involved in regulating the CNS myelination timing (Palazuelos, Klingener, & Aguirre, 2014) and excitatory/inhibitory synaptic input which involves dopaminergic and GABAergic neurons, a quality that supports the potential role of TGF- β1 in neuropsychiatric disorders (Luo et al., 2016). In addition, TGF- β1 has been shown to be involved in regulation of cellular proliferation, cell differentiation, and cell survival. While TGF- β1 is found exclusively within choroid plexus epithelial and meningeal cells, it has been demonstrated to be increased in amount and spread as a response to stresses like ischemia (Pal, Lovas, & Dobolyi, 2014). Yet, TGF- β1 has failed to be considered as a marker for inflammation (Masuda et al., 2017). Microglia polarization, from M1

to M2 is another quality of TGF- β1 that has recently been studied (Xie et al., 2018).

Low Field Magnetic Stimulation (LFMS), a novel approach

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive brain stimulation method widely used in treating major depression and other neuropsychiatric disorders (Brunoni et al., 2017). rTMS is documented to alleviate symptoms like spasticity and depression in MS, but the effects of this brain stimulation on CI is far from clear (Amatya, Khan, La Mantia, Demetrios, & Wade, 2013; Palm, Ayache, Padberg, & Lefaucheur, 2014).

Figure 1.1 Illustration of LFMS device and parameters

A **B**

C

D

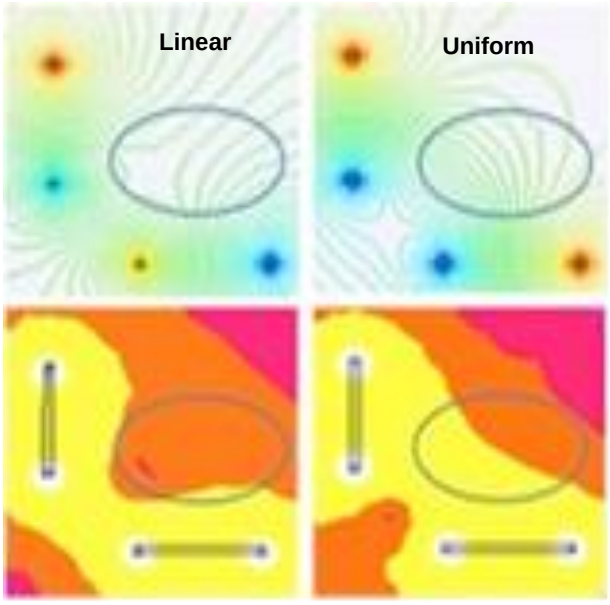


Figure 1.1 A. Illustration of DMS. **B.** Illustration of treatment position. **C.** Magnetic field cluster. **D.** Magnetic field distribution.

Figure 1.2. The schematic diagram of low-field magnetic stimulation (LFMS)

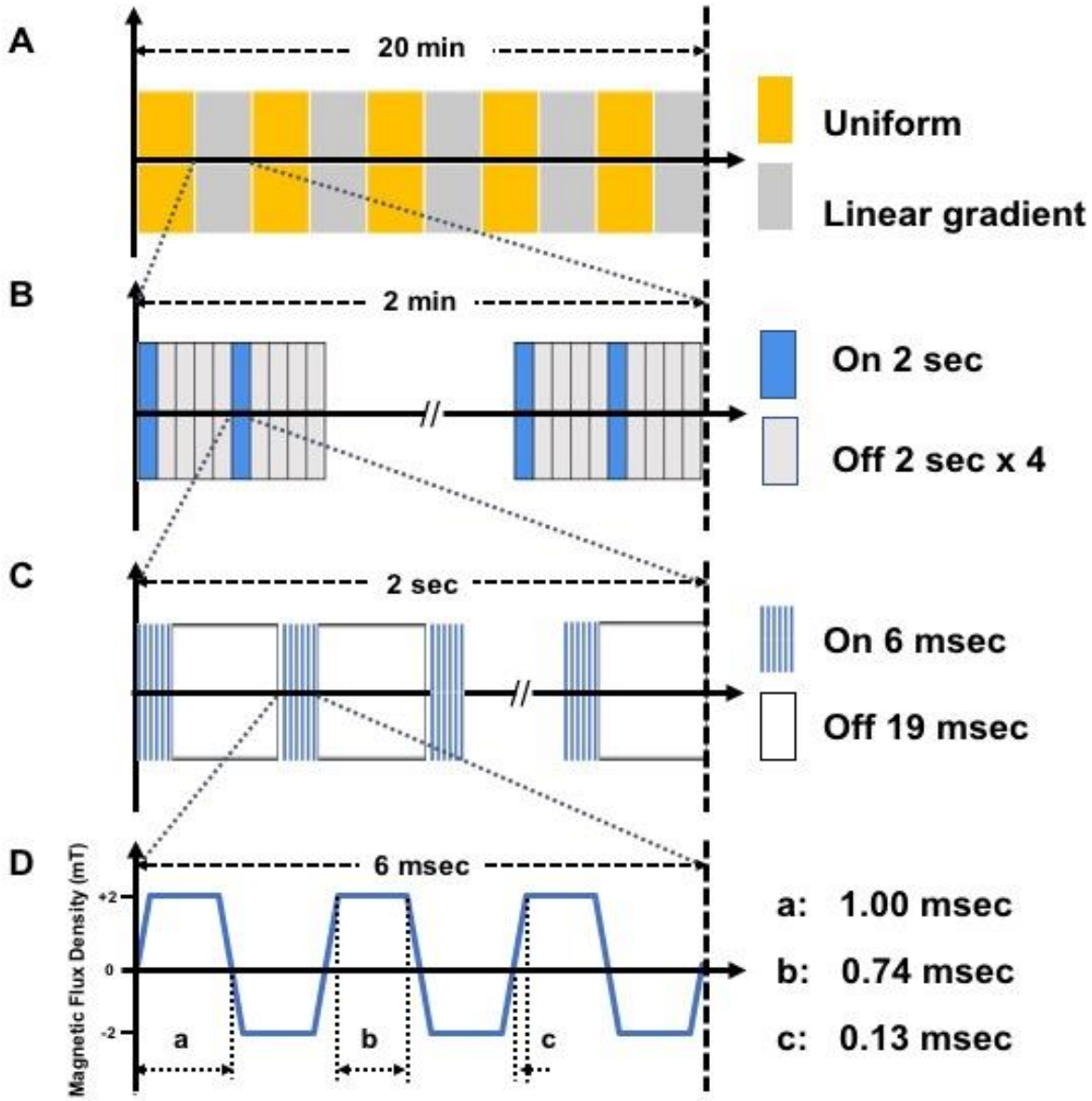


Figure 1.2. The schematic diagram of low-field magnetic stimulation (LFMS). A. magnetic field distribution. Every 2 min the magnetic field is switched between uniform and linear gradient distribution. B. Magnetic pulses. Every 2 seconds output is followed by 8 seconds resting interval. C. In each 2-second stimulation is composed of rhythmical trains spiking 6 ms pulses with intervals of 19 ms and constitutes the intermittent gamma burst stimulation at 40 Hz rhythm. D. Magnetic flux density.

LFMS is a non-invasive magnetic stimulation device. It works through producing diffuse and low-intensity magnetic stimuli to multiple cortical areas (with a magnetic flux density of Max (B) < 5mT, induced electric field Max E < 0.5 V/m) (Shafi, Stern, & Pascual-Leone, 2014) (Figure 1). This magnetic field is less than what is required for inducing neuronal depolarization; however, it is sufficient to induce membrane changes in the cortical and subcortical areas (Shafi et al., 2014). From a clinical aspect, LFMS was shown to be beneficial in treating mood disorders, namely bipolar depression and major depressive disorders, with few treatment-emergent adverse events (Carlezon et al., 2005; Rohan et al., 2014). In animal studies, treatment with gamma band (gamma-LFMS) at 40 Hz showed improvement in depressive symptoms and CI (Yan Zhang et al., 2014; Zhen et al., 2017). It is still unclear how LFMS induces these effects. Gamma-LFMS, similar to other forms of gamma stimulations, applies strong neuroprotective effects by restoring excitatory/inhibitory balance. It also regulates neurogenesis and microglia activation (Yan Zhang et al., 2014; Zhen et al., 2017). Moreover, LFMS may also affect migration and proliferation of oligodendrocyte precursor cells (OPC), which is thought to be regulated through glutamate (Gautier et al., 2015) and GABAergic pathways (Zonouzi et al., 2015)

CHAPTER 2. STUDY DESIGN AND METHODOLOGY

Animal model

Cuprizone (CPZ) is a Copper Chelator which has long been used to induce demyelination (Jelle Praet et.al. 2014) When CPZ is mixed with the animal chow, demyelination will happen in specific areas of the brain, namely, the corpus callosum, frontal cortex, hippocampus, and cerebellum. Acute CPZ demyelination models of MS was used to examine the impacts of gamma-LFMS on 1) CI symptoms associated with demyelination; 2) demyelination and neuronal death; 3) Study the possible role of TGF-β1 in LFMS mechanism of action. To test these hypotheses, C57BL/6 mice (7 weeks old) were purchased from Charles River (Montreal, Canada) and after one week of acclimatization were randomly assigned into 1 of 4 groups in a 2 X 2 experimental design, consisting of CPZ-treated mice receiving either sham treatment (CPZ) or gamma-LFMS treatment (CUPT), and healthy mice with a normal diet who received either sham treatment (CTL) or gamma-LFMS treatment (LFMS) (total N=120). Cuprizone (CPZ, Sigma-Aldrich, St. Louis, MO, USA) was mixed into the milled Lab Diet rodent chow (PMI Nutrition International LLC, Brentwood, MO, USA) with a final concentration of 0.2% (w/w). Food was dispensed between animals twice a week ad libitum because we did not intend to add food restriction to our model as an additional stressor factor. Animals were weighed at the beginning of the experiment to provide us with a baseline weight and then weighed weekly. The weight records were afterwards used for two main reasons. First, weight was used as a general indicator of the animals' condition, since a dramatic decline in weight could be a sign of impending deterioration of health status. Second, CPZ is notorious for causing weight loss in C57BL/6 mice, although weight will start to be regained after the CPZ diet is switched to normal

diet (Praet, Guglielmetti, Berneman, Van der Linden, & Ponsaerts, 2014). All CPZ mice were fed with chow including 0.2% CPZ for up to 6 weeks to induce acute demyelination. At the end of week 3 and week 6, half of the mice from each group underwent behavioural tests followed by euthanasia and pathological studies. The reason behind this time point selection was our aim to study the effects of LFMS on our demyelination model at two different situations, namely: After 3 weeks of CPZ diet which marks the peak of inflammation in the brain and at the end of 6 weeks which is the established complete demyelination. We assumed that comparing the efficacy of LFMS at these time points could provide us with a clue as to the optimum time of the LFMS intervention.

LFMS treatment

Mice in their (non-metal) cages were put on the LFMS device and received 20-min sham or LFMS treatment from day one of the experiment, on a daily basis (excluding the weekends), until the last day that the mice were in the experiment. The device is made of two 360 mm-diameter-coils and a control centre that generates intermittent gamma band stimulations (Figure 2). The settings of the machine were based on previous studies (Yan Zhang et al., 2014; Zhen et al., 2017). In brief, the magnetic field alters every 2 min between approximate and linear gradients. The alternation cycles include 2 seconds (sec) on and 8 sec off and the treatment was applied for 20-mins. Every 2-second-output is made of 80 trains of LFMS, which forms a 40-Hz rhythm. There are 6 pulses in each train at 1000 Hz frequency and 19 msec intervals. The Maximal magnetic flux density (B_{Max}) is less than 2 mT, with peak induced electric field (E_{Max}) of less than 0.5 V/m (Figure 3). The mice in the sham group followed the same treatment routine except for receiving no magnetic stimulation.

Behavioural tests

Motor function

In order to make sure that the results of the behavioural tests are a true reflection of the cognitive state of the animals**,** the animal movements in Open Field Test (OFT) and Y-maze were recorded and analyzed (please see the details below).

Open field test

The anxiety-like behaviour of mice was assessed in an OFT for 5 min (He, Xu, Yang, Zhang, & Li, 2005). The OFT was performed using a roofless cubical (50x50x40 cm) made of white hard plastic sheets located in a well-lit silent room (Figure 3). The surface of the box floor was divided into five equal squares and the outer row of these squares was determined as the peripheral area and the rest of the nine squares in the middle were appointed as the central area (Michael L. Seibenhener et. al. 2015). For each animal to be in one of these areas, more than 90 percent of the animals' body must have been inside that area. The OFT works based on the mouse's constant struggle between the urge to explore new areas and their paucity of assertiveness as small rodents. The conflict between the desire to explore and the desire to avoid the anxiogenic stimuli of open and high spaces in rodents is a useful tool to distinguish whether an animal's level of anxiety has reached a point that leads to a significantly less exploratory behaviour (Sumnall, O'Shea, Marsden, & Cole, 2004). The mice were placed at the center of the box and left free to explore the area for 5 min and their movements were recorded during the test time. The time that the animal spent in the center of the box during the test was used as an index of the anxiety level of the animals. Moreover, the distance travelled by each mouse and the lines

they crossed were also calculated using ANYMAZE software and used as a measure of the animal's gross motor function.

Y-Maze

The Y-Maze is a gross test for spatial memory (Lamberty Y, 1992). It is a well-known test which is usually used for two different aspects of the cognition: spatial working memory and recognition memory. It consists of a Y formed apparatus with three equal arms each 120˚ apart from each other and 40 cm in length, 5cm wide and 10 cm in height. To measure the animals' intermediate to long term recognition memory, a two-trial mode of Y-maze (also called forced alternation test) is performed, (A Comprehensive Behavioral Test Battery to Assess Learning and Memory in 129S6/Tg2576 Mice, Andrea Wolf et. al. 2016) meaning that at first the animal is trained to roam the maze while one of the arms is blocked with an object inside and after a few training sessions, the closed arm is opened, and the mouse is allowed to explore the whole maze. It is expected that the animal dedicates more time to visiting the newly opened arm which was previously closed. However, what we measured in our animals was spatial working memory which is usually assessed by checking the percentage of complete spontaneous alternations. Each mouse was place at the end of an arm and in order to preserve the consistency of the test, arm "A" was chosen as the start point of the Y-maze to begin with and mice were allowed to explore all three arms freely. Each time that a mouse completes a set of three non-repeating entries to the maze's arms was calculated as one alternation and the whole test was recorded for 5 mins (Joyce L. W. Yau et. al. 2007 & Mehrdad Faizi et. al. 2011). This Y-maze test assesses the ability of the mouse to remember which arm they entered last and pick a different one for their next arm entry, a value that is calculated as the percentage of the spontaneous alternations

completed during the test period. The number of entries to each arm was also calculated and used as a measure of the animals' mobility. The total number of entries minus 2 was determined as the maximum potential number of alternations. Then the real occurred alternations were calculated and normalized as the percentage of total possible alternations. The criteria for the animal entry to be considered as a real entry into the arm was when the whole animal's body (including at least two thirds of the tail) was inside the arm. If an animal had less than eight entries during the whole course of the test, that animal was excluded from the group. If a mouse re-entered an arm right after exiting it but before entering another arm, it was not counted as a separate entry and was not included in calculation formula for the total possible number of alternations of the animal.

Forced-swimming test (FST)

This test induces a "behavioural despair" that comes from being in an unescapable situation. The FST is used as a predictive tool to posit the probability of a therapeutic intervention efficacy for anti-depressant treatments. The cumulated time that the mouse is not actively attempting to escape during the test indicates "behavioural despair" and is calculated as total immobility time. The time that it takes for the animal to actually show the first sign of despair, which is when is starts to float with no struggle in the test jar, is recorded as "The latency to the first immobility" (Steru, Chermat, Thierry, & Simon, 1985). In FST, the mice will be placed in a Plexiglas cylinder (10 cm internal diameter, 20 cm high) filled with 10 cm deep 25–26 °C water. The duration of the experiment is 6 min, and the immobility of mice will be counted during the last 5 min (Porsolt, Le Pichon, & Jalfre, 1977). We did repeated FSTs to check the ability of the mice to habituate. This marks a basic form of learning in animals, on the

theory that animals with a normal cognitive state should be able to adapt to new conditions faster than those who suffer from cognitive impairment. When exposed to a stressful, yet non-fatal situation, we expected the mice in the CTL group to show signs of adaptability earlier than those with CI, as it has previously been documented that normal mice tend to do so (Mul, Zheng, & Goodyear, 2016).

Brain histology and immunostaining

Euthanasia and tissue preparation

Following each of the behavioural test batteries, half of the mice from each group were randomly selected for euthanasia and tissue harvest. Animals were anesthetized in Isoflurane chamber and then perfused transcardially while still under the influence of Isoflurane. In brief, the animal's thoracic cavity was exposed and using a sharp pointed pair of scissors, a nick was made in the right atrium. Then 2-4% Phosphate Buffer Saline (PBS) was slowly perfused into the left ventricle. The animal liver's color was used as an index of perfusion success and when the liver color became significantly pale, 4% Paraformaldehyde (made from scratch using Cold Spring Harbor Protocol) was perfused to fix the brain. (Tissue designated for Western Blot samples were harvested without PFA perfusion) The brains were then carefully extracted and dissected into different areas namely, prefrontal cortex, middle cortex, rest of the cortex, striatum, cerebellum, and hippocampus. Brain areas were cut in part by using a Plexiglas brain matrix and according to Allen Brain Atlas. Western blot samples were kept in 2mm tubes in -80˚C and samples meant for IHC and IFF were kept in 4% PFA until after 24 hours, when they were rinsed using 2-4% PBS and kept in PBS until the day they were used.

Myelin pathology

Imaging analysis Pic Integrated density of myelin basic protein (MBP) immunostaining in unified areas of the brain sections was used to identify cortical and hippocampal myelin tracts and GM demyelination (Mei et al., 2012; Y. Zhang et al., 2008). Pictures were selected and uploaded into Image j software. Before starting the quantification, the software was calibrated using an optical density step tablet downloaded from Stoufer industries as per Image J recommendation in the online tutorial (https://imagej.nih.gov/ij/docs/examples/calibration/). Then the picture type was changed into 8 bit in order to provide us with a black and white image, which was then edited using the threshold option. Threshold for the color density was set on auto in order to minimize the researcher bias. Areas from the pictures were selected (the same surface from each picture was selected) and the integrated density of the image was calculated and used as the value for comparing the accumulated density of myelin fibers in the selected areas.

Western blot

Western blot was used in order to study the levels of different proteins in the brain samples (McLean & Verge, 2016). In brief, after the removal of the brain, one hemisphere from randomly selected mice in each group was dissected into the prefrontal cortex, rest of the cortex, striatum, cerebellum, and hippocampus, to be frozen and stored at −80 °C until further use. For Western blot procedure, the brain samples were homogenized and then centrifuged for 10 minutes at 4˚C and 1500 RPM. The supernatant was separated and pipetted into 2 ml tubes and stored at −80 °C. Bradford assay was used to quantify the protein amount in each sample and then X4 loading buffer was added to the samples. At this point, each sample tube was boiled for 5 minutes and afterwards stored at −80 °C for future use in blotting assay. Bio-Rad TGX

miniprotean pre-cast gels were used for Western blot procedure. Gels were placed inside the running tanks (Bio Rad) and loaded with the unified amount of prepared samples. (A total of 30 µg of protein was loaded into each well. The gels were run with 120 V of current (90V at the beginning, which was increased to 120 as soon as the ladder colors started to separate). After 60 minutes the gels were collected and used to make sandwiches for the transfer procedure. Each sandwich was made using the following order of the material: sponge, filter paper, gel, membrane, filter paper, sponge and the tank was filled with transfer buffer (1/10 of 10X transfer buffer + 8/10 DD water+ 1/10 Methanol) and transferred with 100 V for 75 minutes at room temperature. The membranes were then taken out and washed with Double Distilled (dd) water. Next, the membranes were blocked using 5% milk for one hour and then washed with TBST three times, each time for 5 minutes. Primary antibody was diluted in 5% BSA and left at the cold room (4˚C) overnight for incubation. The next day, membranes were taken to the room temperature and washed for three times, each time for 5 minutes. Afterwards, the secondary antibody was incubated and after 1 hour the membranes were washed and proteins were visualized with enhanced chemiluminescence using the Bio Rad imaging machine. Different antibodies were chosen to examine the proteins of interest, namely, MBP to check the myelin expression in brain samples, Glial Fibrillary Acidic Protein (GFAP) which is commonly used as a general marker of Astrogliosis and inflammation and finally TGF-β1 as our protein of interest based on our hypothesis. Beta Actin was used as the loading control protein and all membranes were analyzed based on their relative measurement to beta actin.

Statistical analysis

One or two-way ANOVAs were used based on the experimental design, which was

followed by Tukey's post hoc test or two-tailed Student's t-test for comparing individual means of behavioural and pathological data between different groups in the experiment and the control. The behavioural and pathological data were assessed in two different time points of the experiment (after the completion of week 3 and week 6). The statistical significance was set at $p < 0.05$.

Ethical approval

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

CHAPTER 3. RESULTS

No differences in motor function tests throughout acute CPZ model

Distance travelled by the animals in OFT results yielded no significant difference between groups either at 3 weeks or at 6 weeks of the study (Figure 3) ($P>0.05$ in two way ANOVA analysis followed by serial T-tests). Distance travelled during the Y-maze was also not significantly different between groups at the same time points (Figure 7) ($P>0.05$ in two way ANOVA analysis followed by serial T-tests). In addition line crossing in OFT and Y-maze showed no significant changes between groups (figures 5 & 8). Total time immobile during OFT was also not significantly different among study groups in both time points (Figure 6). In Y-maze, mean speed of the mice was also calculated by the software and did not show any significant differences between groups at any time during the study (Figure 10).

OFT revealed different time spent in the center among groups

After 3 weeks of the experiment the (Figure 4), **–CPZ/+LFMS** group showed a significantly longer time spent at the central area of the box when compared to the CTL group ($P<0.05$). At the same time both other groups namely, **+CPZ/-LFMS** and **+CPZ/+LFMS** also spent a significantly longer time at the center. ($P<0.05$) and there were no significant differences between the two latter groups ($P>0.05$). When the mice were tested after 6 weeks, the CPZ fed groups still showed a significantly longer time at the center ($P<0.05$ and $P<0.005$ for **+CPZ/-LFMS** and +**CPZ/+LFMS** respectively), while the **–CPZ/+LFMS** group demonstrated a dramatic decline in the time spent in the central area, although significantly less than **CTL** ($P<0.05$).

More significant drop in spontaneous alternations after 3 weeks than 6 weeks of CPZ diet

Y-maze results (Figure 7) at the end of the 3 weeks showed a significantly lower percentage for spontaneous alternations of the **+CPZ/-LFMS** mice compared to **CTL** ($P < 0.0001$). Animals that received CPZ diet but no LFMS (**+CPZ/- LFMS**) showed more dramatic changes in their alternation percentage after 3 weeks than 6 weeks of CPZ diet ($P<0.0001$ vs. **CTL** after 3 weeks compared to $P<0.05$ vs. **CTL** after 6 weeks).

LFMS improved the Y-maze findings both after 3 weeks and 6 weeks in C57/bl6 mice

The mice who received LFMS treatment along with the CPZ diet manifested less significant drop in their spontaneous alternations after 3 weeks of CPZ when compared to **+CPZ/-LFMS** group at the end of the third week. ($P<0.0001$ for **+CPZ/-LFMS** and $P<0.05$ for **+CPZ/+LFMS**) Yet, the **+CPZ/+LFMS** group was not significantly different when compared with the **+CPZ/-LFMS** group at this point ($P=0.0773$). When tested after 6 weeks, not only did the **+CPZ/+LFMS** group show significantly higher results in spontaneous alternation compared to **+CPZ/-LFMS** ($P<0.05$), but their score was not significantly different from the CTL ($P=0.173$). Among all the Y-maze sets of results, there was no significant difference between CTL/Sham and CTL/LFMS groups ($P=0.153$ after 3 weeks and $P=0.421$ after 6 weeks) (Figure 7).

LFMS altered the results of Forced Swim Test (FST) for the naïve mice in CPZ model

During a standard Forced Swim Test (FST) after the first three weeks of the experiment (Figure 11A), the **+CPZ/-LFMS** group demonstrated a significantly longer *total immobility time* compared to **CTL** ($P<0.01$). At the same time, the **+CPZ/+LFMS** results were not significantly different from the **CTL** group ($P=0.354$) and were significantly different from **+CPZ/-LFMS** ($P<0.05$). Moreover, when the *latency to the first immobility* was recorded, **+CPZ/-LFMS** group were significantly different from the **CTL** ($P<0.01$). While **+CPZ/+LFMS** mice were not different from **CTL** ($P=0.421$), they showed a significantly different result than those who were not treated with LFMS ($P=0.037$). (Figure 14)

Multiple FST revealed a specific behavioral pattern in CPZ model

During the second FST (after 24 hrs from the first one), the **CTL** showed an increase (although not significant, $P=0.18$) in their total immobility time, while the **+CPZ/-LFMS** group demonstrated a significant lower immobility time than the first FST ($P=0.0005$) (Figures 11-B, 12, and 13). When the animals in **+CPZ/-LFMS** group were re-tested after 3 more weeks of CPZ diet (Figures 11-C, 12, 13) , their total immobility time was even significantly less than what it was before ($P=0.0319$). In contrast, the **CTL** group had significantly higher immobility time ($P=0.024$). The results of the last day of FST did not show any significant changes in any of the groups except for **+CPZ/+LFMS** ($P=0.0282$). The figure for the latency to the first immobility episode also revealed interesting results (Figure 14). When retested after 24 hrs from the first FST, all groups had significantly lower latency times compared to the first day;

however, **+CPZ/-LFMS** group had a significantly higher value compared to the **CTL** (P=0.037). The same pattern was observed during the third FST for CPZ fed mice who received no LFMS (P=0.032). It was only in the fourth FST that the **+CPZ/-LFMS** group finally showed a latency time that was not significantly higher than other groups.

LFMS altered the behavioral response of the mice on CPZ diet during multiple FSTs

During the first FST retest, **+CPZ/+LFMS** group did not show a significant drop in total immobility time (P=0.155), unlike the **+CPZ/-LFMS** group (P=0.0005) (Figure 11-A). After 3 more weeks, although the treated group was significantly different from the CTL group (P<0.0001) (Figure 11-C) they showed significantly better results than **+CPZ/-LFMS** group (P=0.022). At the last round of FST (Figures 11-D, 12, and 13) the only group that showed a significant increase in total immobility time was the **+CPZ/+LFMS** (0.0282) group. In terms of latency to the first immobility episode, the **+CPZ/+LFMS** group showed no significant differences from the **CTL** group in the second round of FST (P=0.395) (Figure 14) and had significantly better results than **+CPZ/-LFMS** group (P=0.0371). The LFMS treated mice also showed no significant difference from the **CTL** (P=0.084) in latency time when tested after 3 more weeks.

LFMS Changed the demyelination state in CPZ model

The Immunohistochemistry results of myelin basic protein (MBP) staining revealed demyelination in frontal cortex and hippocampus areas in CPZ fed mice in both time points. After 3 weeks, the **+CPZ/-LFMS** and **+CPZ/+LFMS** groups both showed significantly lower

integrated density of MBP staining in frontal cortex compared to **CTL**. ($P<0.0001$ and $P<0.001$ respectively) (Figure 18). However, the **+CPZ/+LFMS** group showed significantly higher density compared to the non-treated group **+CPZ/-LFMS** ($P<0.05$). In the hippocampus area the significant difference between both groups with CPZ diet and **CTL** was similar ($P<0.0001$), while no significant difference was found between **+CPZ/-LFMS** and **+CPZ/+LFMS** (Figure 19). Based on the findings from the same immunostaining methodology after 6 weeks (Figure 20) the **+CPZ/-LFMS** mice were significantly demyelinated in the frontal cortex area compared to the **CTL** ($P<0.0001$); whereas, the **+CPZ/+LFMS** group showed less significant demyelination when compared with **CTL** ($P<0.044$) and interestingly, significantly different when compared to **+CPZ/-LFMS** ($P=0.019$). The results for hippocampus area after 6 weeks (Figure 21) showed a significant difference between both **+CPZ/-LFMS** and **+CPZ/+LFMS** groups compared to **CTL** ($P<0.0001$) and there was no difference between the two groups who had received CPZ diet. Based on the IHC results the most significant difference between the CPZ fed mice who did not receive LFMS treatment and those who did was observed in the frontal cortex area at the end of the 6 weeks (Figure 22). This observation was also double checked by Western blot as another semi-quantitative method (Figure 16). The results showed that **+CPZ/+LFMS** group had significantly higher expression of MBP compared to **+CPZ/-LFMS** ($P<0.05$), although both groups were significantly lower than **CTL** ($P<0.0001$). Using the same methodology, the expression of GFAP was measured in the brains that were collected after 6 weeks of CPZ model. There was a significant difference between the two groups of mice with normal chow compared to the mice who were fed with CPZ. ($P<0.0005$) (Figure 17).

CPZ significantly decreased the animal weight.

Animals' weight was monitored and measured at the end of each week. At the end of the fourth week there was a significant decline in the animals' weight in CPZ fed groups and the mice that had received LFMS treatment were not significantly different from the other animals who had normal chow.

LFMS significantly increased TGB-β1 in the mouse brain

Western blot results showed a significant difference between the TGB-β1 expressions in brain tissue of the groups that received LFMS treatment compared to those who received sham treatment. (Figure 17). Two-way ANOVA found a significant difference between the untreated groups and the groups that did not receive LFMS treatment in samples from hippocampus ($P=0.0118$). The same trend was found between the means of groups in the results from frontal cortex after the completion of six weeks of CPZ model, while no significant difference was noticed between groups.

Figures

Figure 3.1: Distance travelled by mice during Open Field Test in two time points

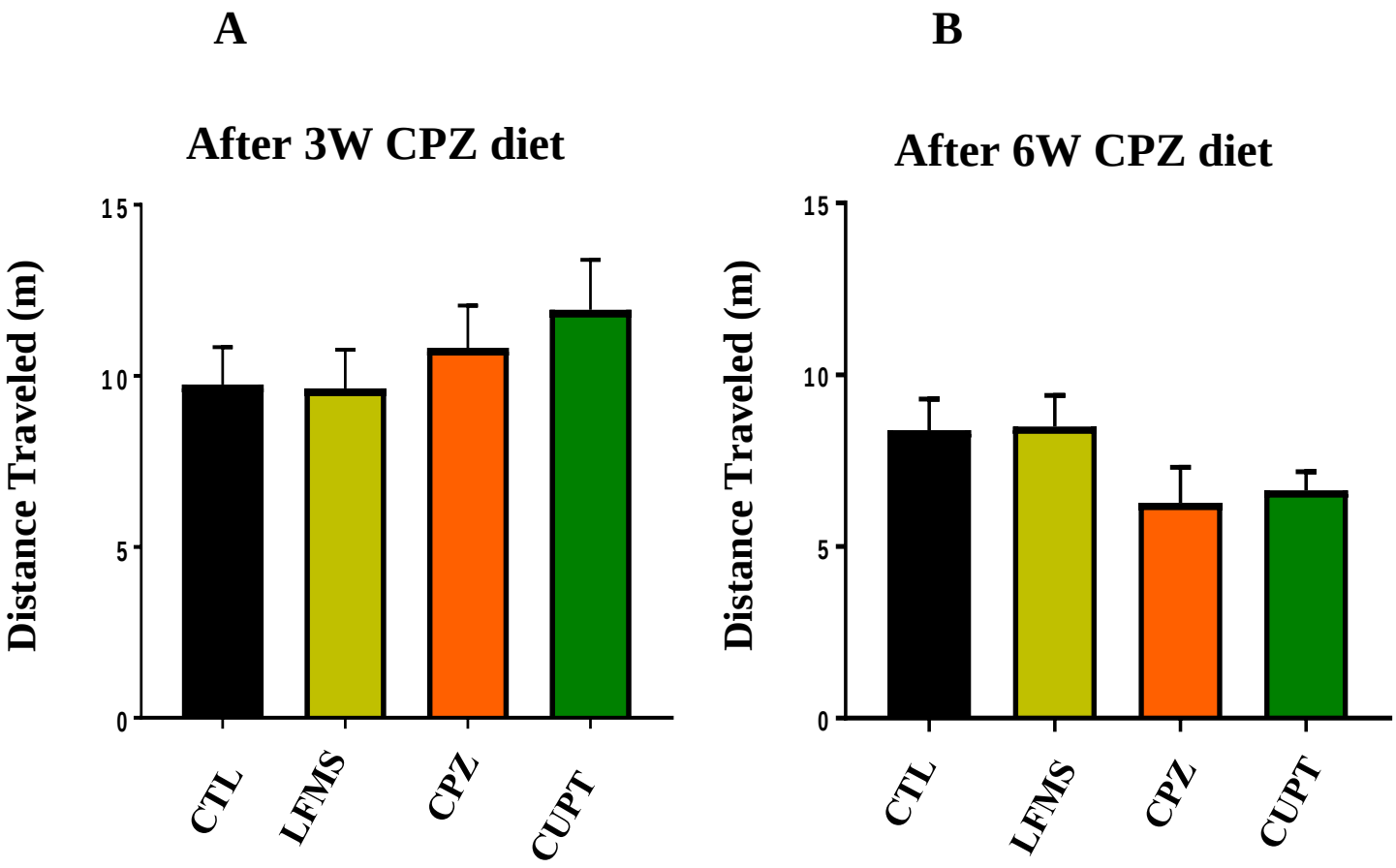


Figure 3.1: Distance travelled by mice during Open Field Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means± SEM (n=10-15 per group).

Figure 3.2: Time spent in the central zone during OFT in two time points

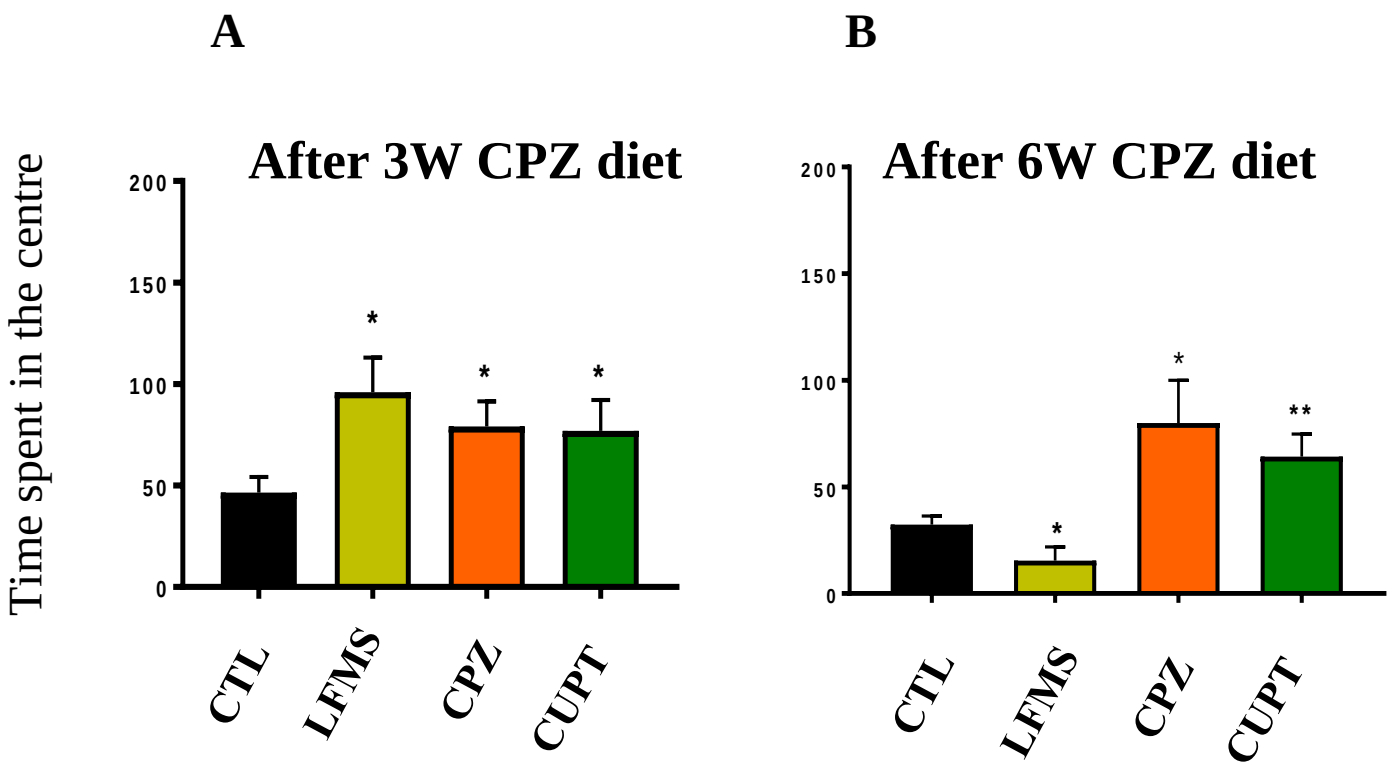


Figure 3.2: Time spent in the center during Open Field Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means ± SEM (n=10-15 per group). (* $p<0.05$ VS CTL) (** $p<0.005$ VS CTL)

Figure 3.3: Line crossing during OFT in two time points

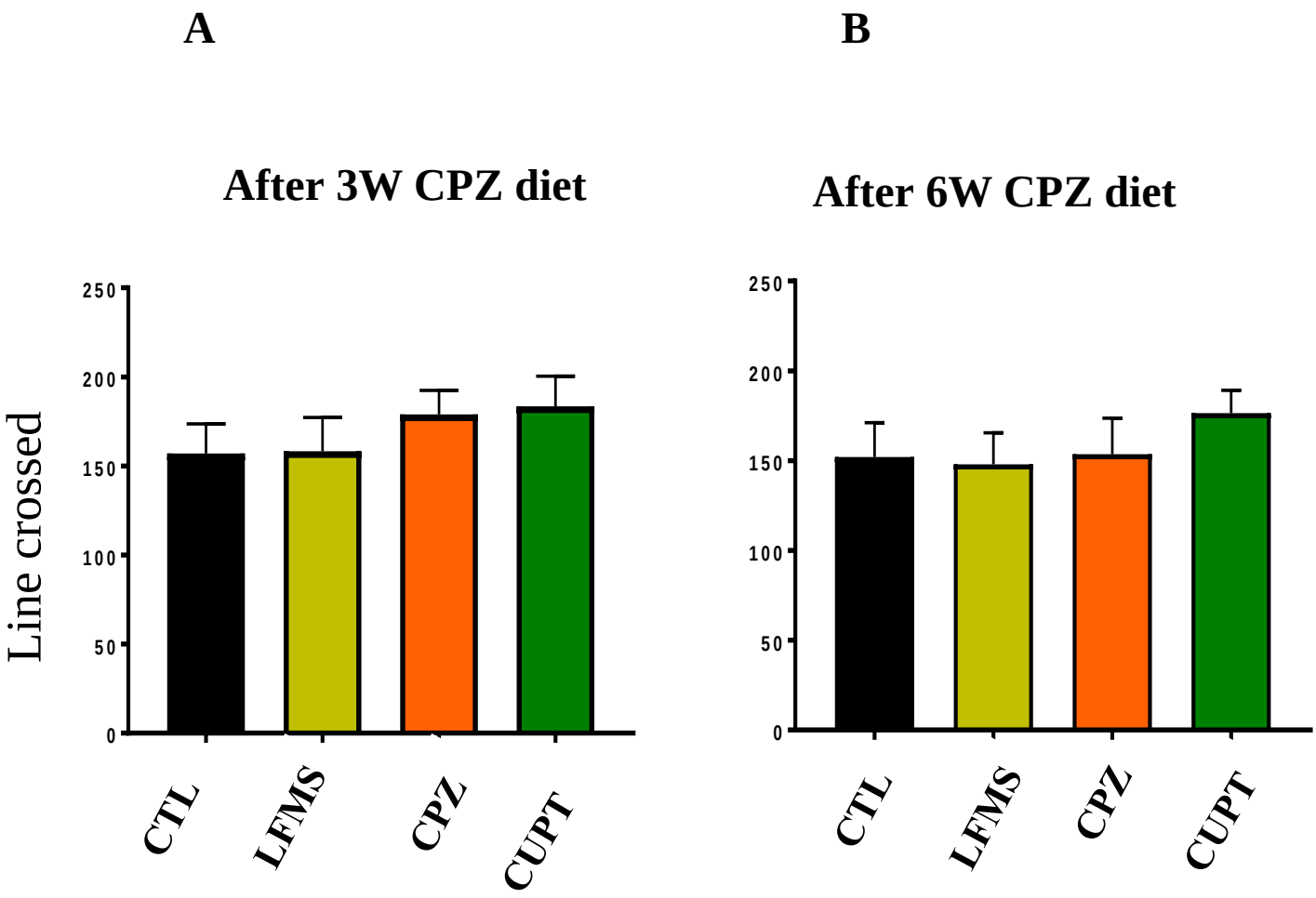


Figure 3.3: line crossing by mice during Open Field Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means± SEM (n=10-15 per group).

Figure 3.4: Total time immobile during OFT in two time lines

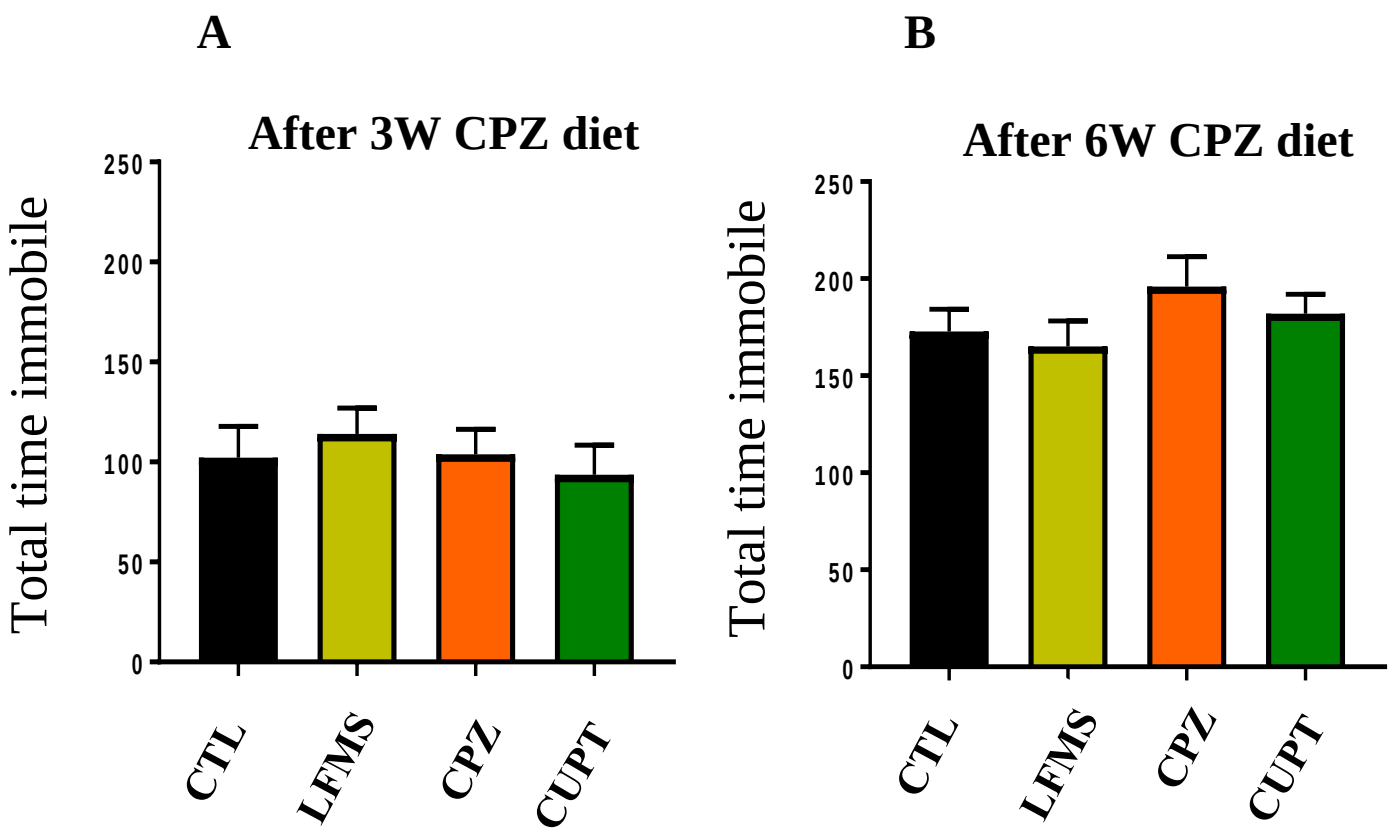


Figure 3.4: Total time immobile for mice during Open Field Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means± SEM (n=10-15 per group).

Figure 3.5: Spontaneous alternation during Y-maze in two time points

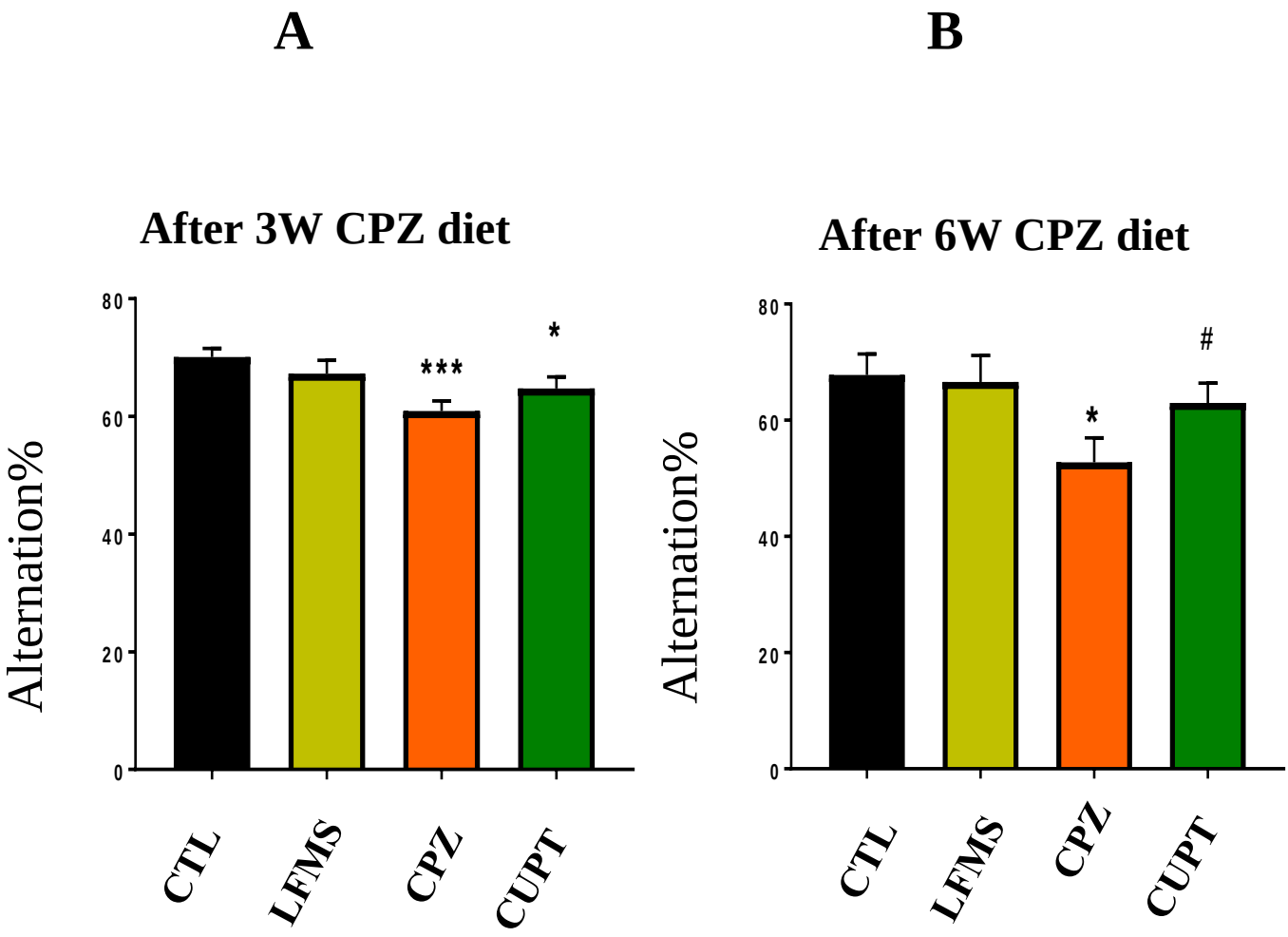


Figure 3.5: Proportion of spontaneous alternations in Y-maze for two time points. (A) Shows the results after 3 weeks of experiment and (B) represents the results after 6 weeks. Data is presented as means± SEM (n=10-15 per group). *p<0.05 vs. CTL, *** p<0.0001 vs. CTL, and #p<0.05 vs. CPZ.

Figure 3.6: Lines crossed during Y-maze in two time points

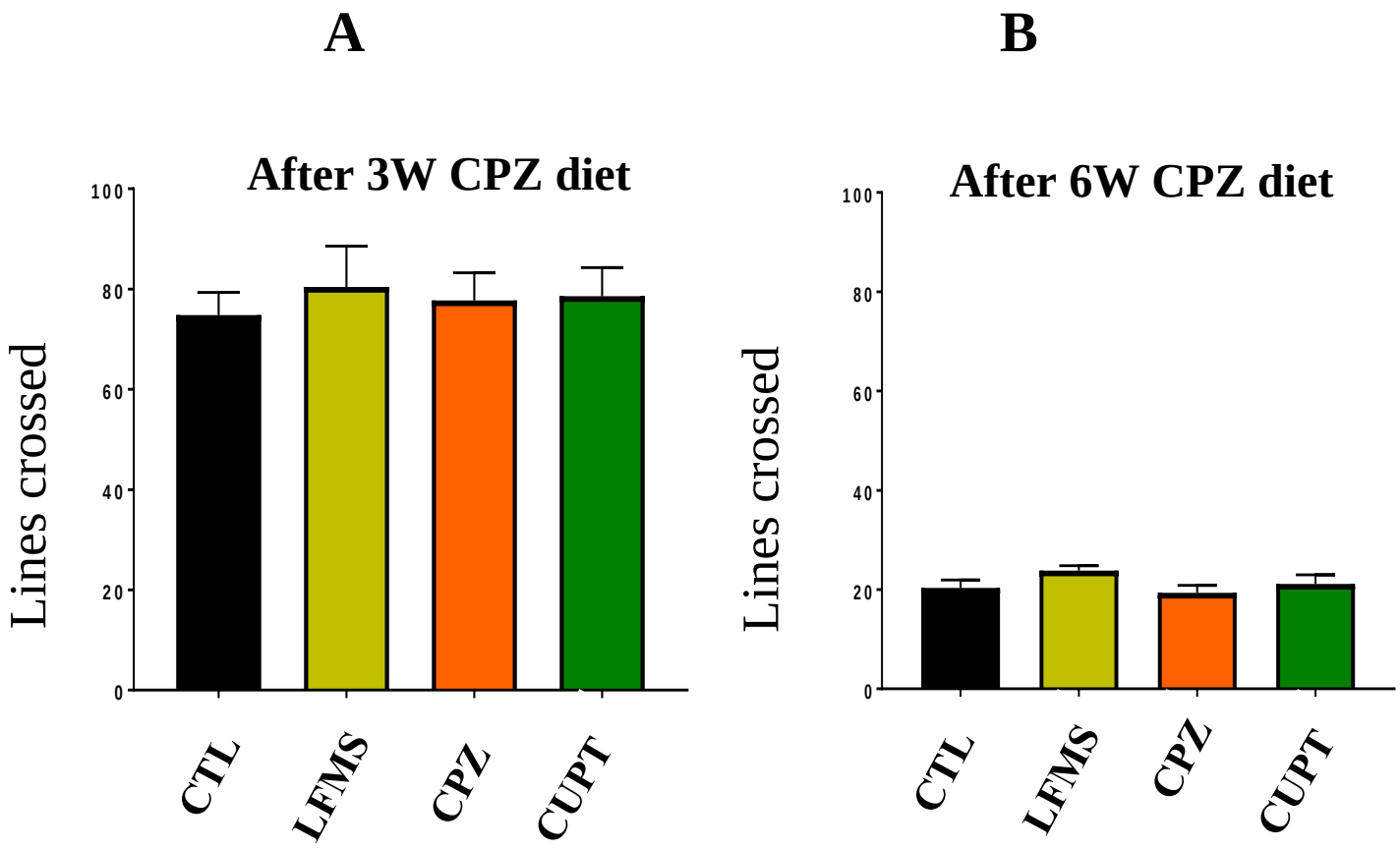


Figure 3.6: Line crossing by mice during Y-maze Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means± SEM (n=10-15 per group).

Figure 3.7: Distance travelled by during Y-maze in two time points

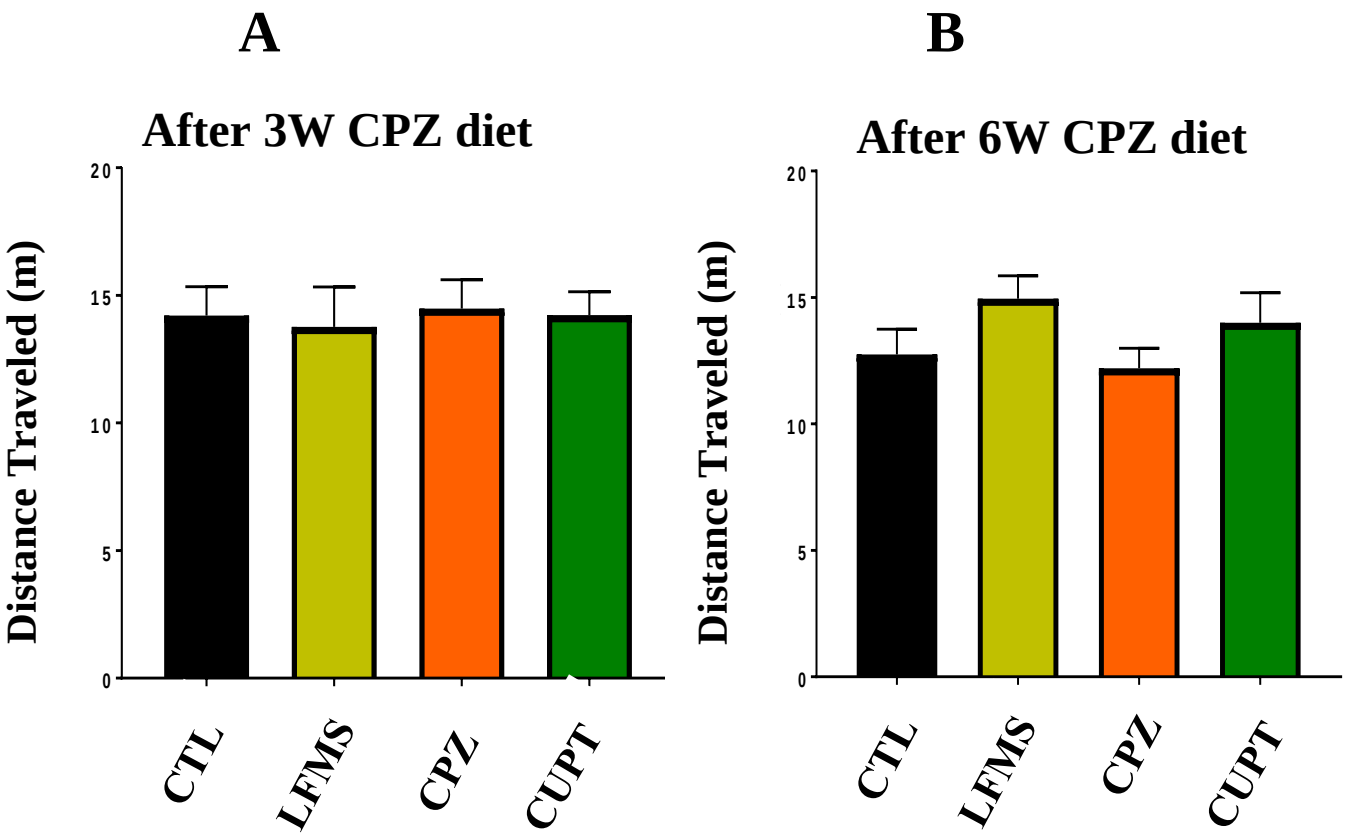


Figure 3.7: Distance travelled by mice during Y-maze Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means± SEM (n=10-15 per group).

Figure 3.8: Mean speed of the mice during Y-maze in two time points

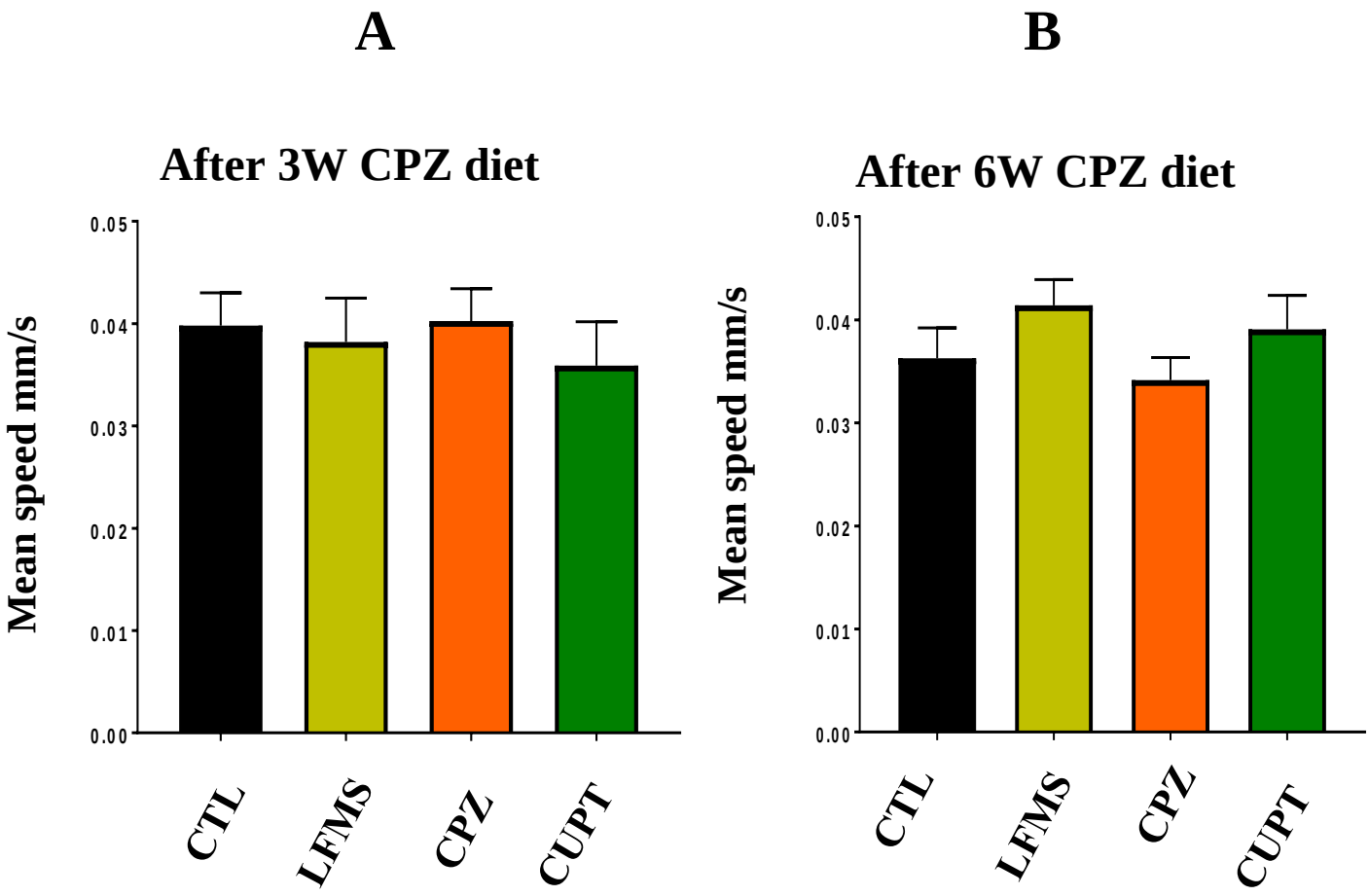


Figure 3.8: Mean speed of the mice during Y-maze Test in two time points. (A) After 3 weeks of experiment and (B) after 6 weeks of experiment. Values are in meter. Data is presented as means± SEM (n=10-15 per group). ($p<0.05$)

Figure 3.9: Total immobility time compared during the four sessions of FST

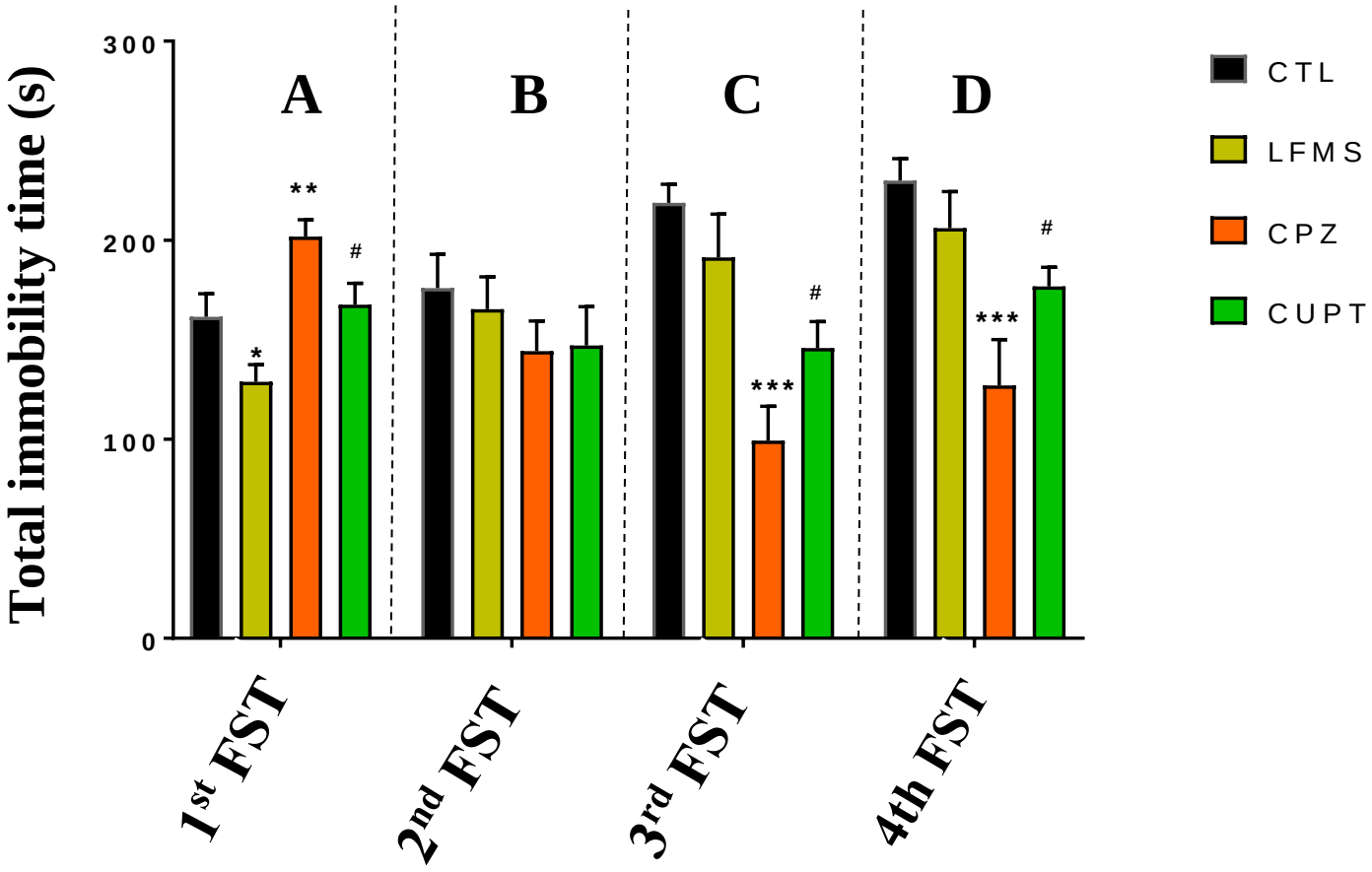


Figure 3.9: Total Immobility time [in seconds] during 5 minutes of Forced Swim Test (FST) in the following 4 time points. (A) Shows the results for the first day [first exposure] after 3 weeks of the experiment. (B) Shows the results of the second FST during the fourth week of the experiment. (C) Represents the results for the third FST after 6 weeks of the experiment. (D) Shows the results for the fourth FST performed before brain collection. Data is expressed as means± SEM (n=10-15 per group). *p<0.05 vs. CTL, **p < 0.01 vs. CTL, ***p = 0.001 vs. CTL, ****p<0.0001vs. CTL, and #p<0.05 vs. CPZ.

Figure 3.10: Total immobility time compared with the previous session of FST through four sessions of FST during 6 weeks of experiment.

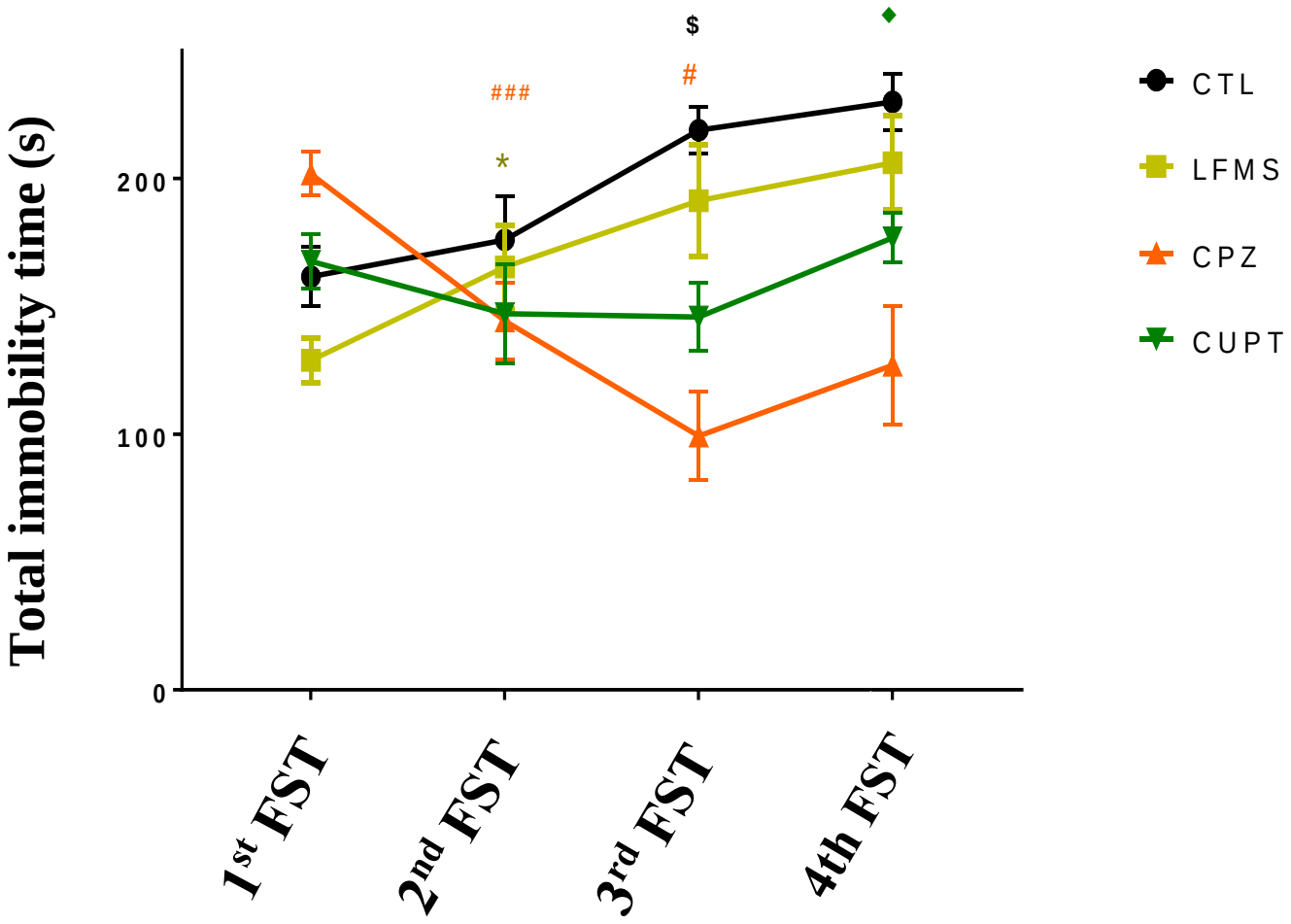


Figure 3.10: Total immobility time in FST through the experiment timeline. Data is expressed as means± SEM (n=10-15 per group). *p<0.05 vs.1st FST, ### < 0.001 vs. 1st FST, # <0.05 vs. 2nd FST, $ p<0.05 vs. 2nd FST, and ♦ p<0.05 vs. 3rd FST.

Figure 3.11: Latency to the first immobility episode through time

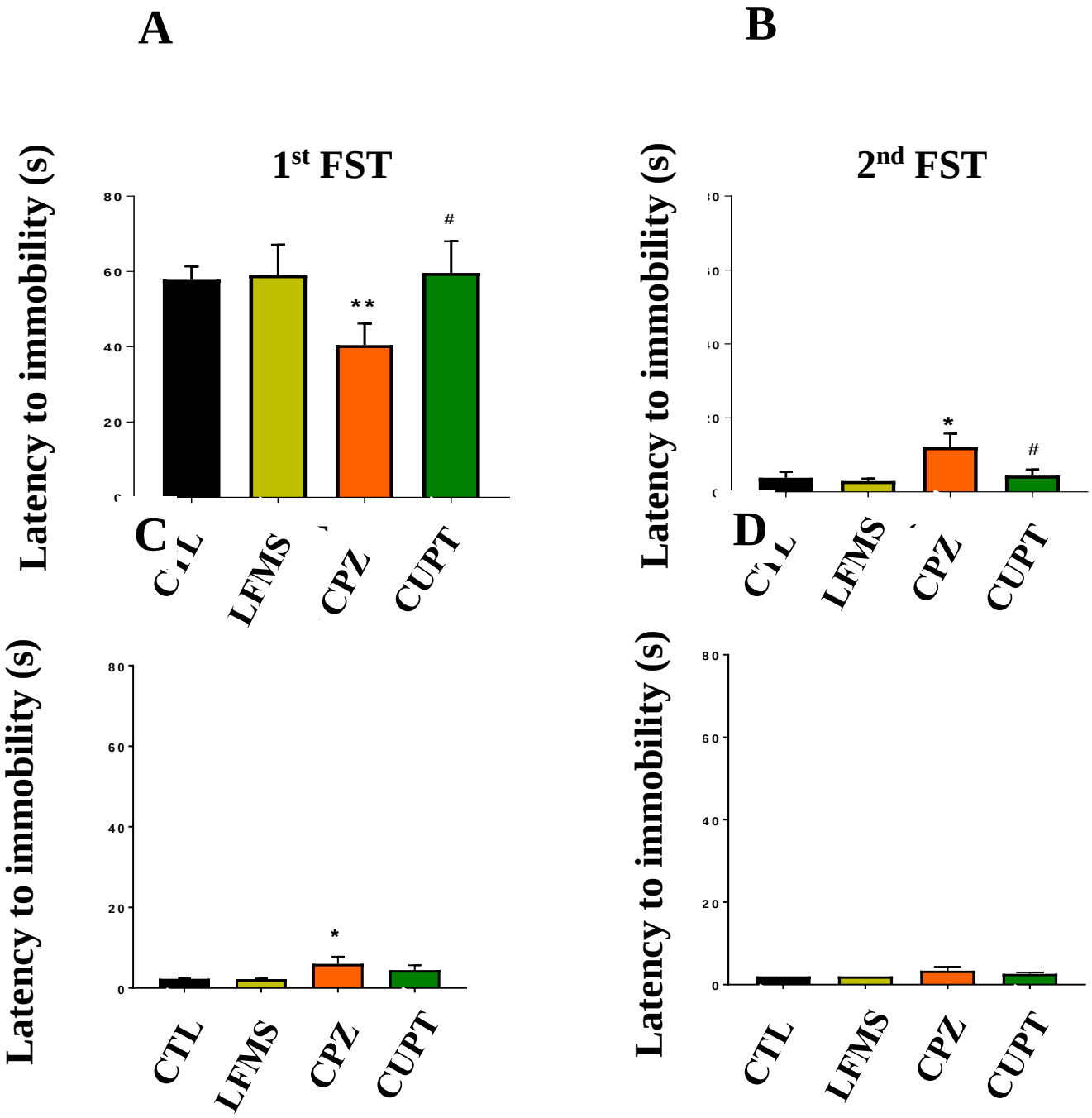


Figure 3.11: Latency until the first immobility period as a result of FST in the following 4 time points: (A) the first FST at the end of the 3 weeks after the start of the experiment. (B) Second FST done in the fourth week. (C) Shows the results of the third FST performed after 6 weeks of experiment. (D) Represents the last FST before brain tissue collection. Data is presented as means± SEM (n=10-15 per group). *p<0.05 vs. CTL, **p <0.01 vs. CTL, and #p<0.05 vs. CPZ.

Figure 3.12: Latency to the first immobility episode compared to previous episodes of FST through 6weeks of the experiment.

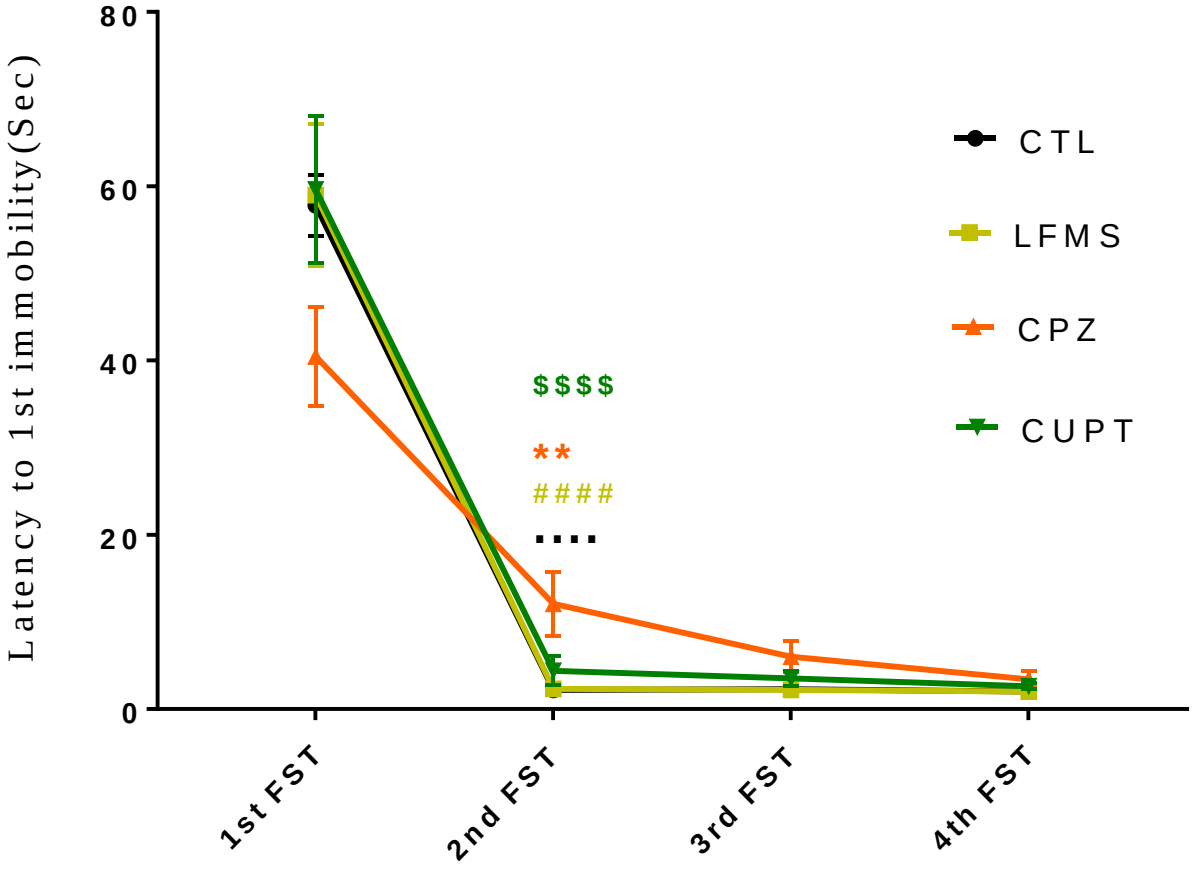
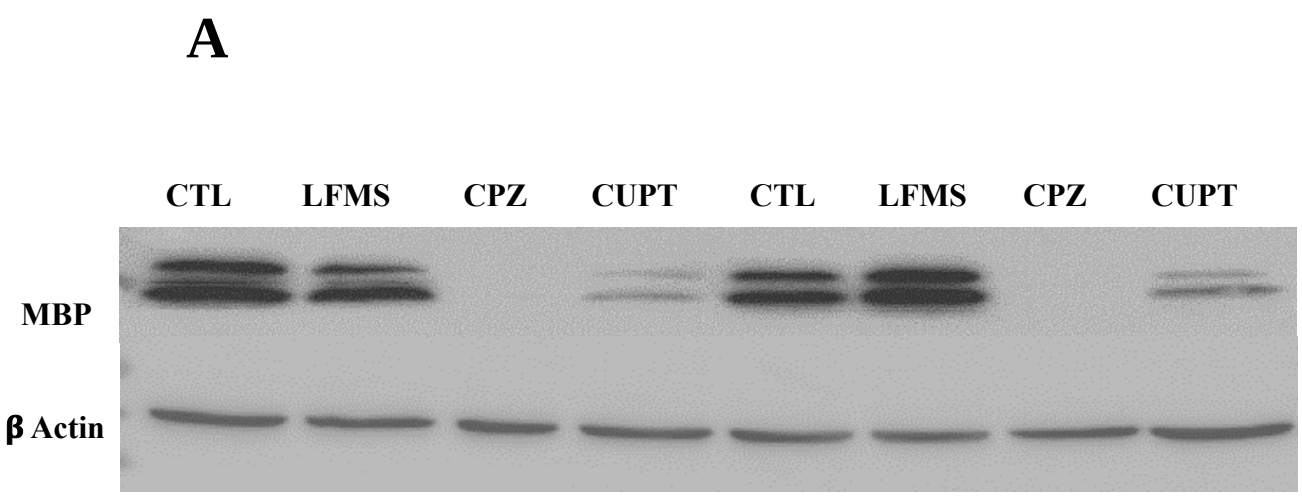


Figure 3.12: Latency until the first immobility period compared by sessions: Data is presented as means± SEM (n=10-15 per group). ▪▪▪▪ p<0.0001 vs. 1st CTL, **p <0.005 vs. 1st CPZ, ####p<0.0001 vs. 1st LFMS, and $$$$ p<0.0001 vs. 1st CUPT.

Figure 3.13: Western blot of Myelin Basic protein after 6 weeks of acute demyelination.



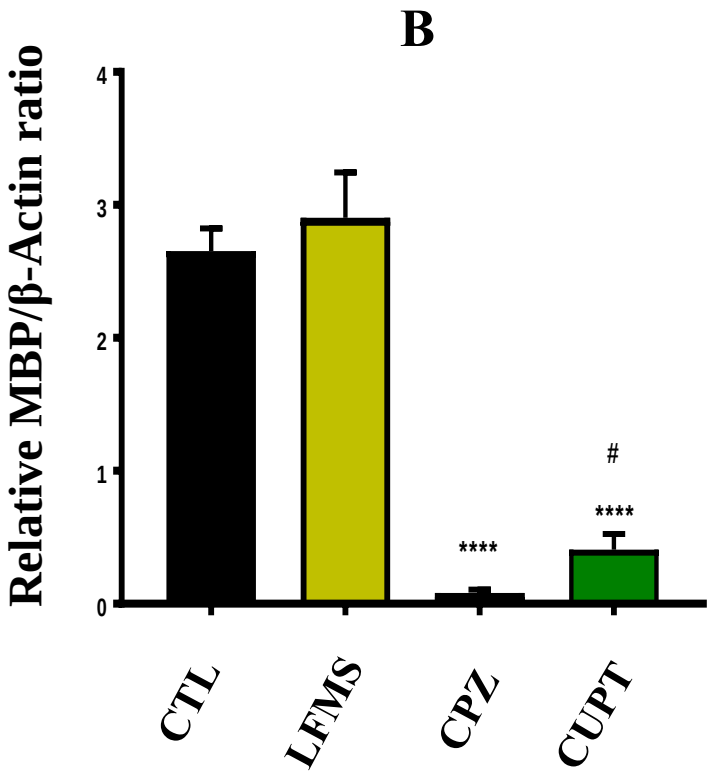


Figure 3.13: (A) Expression level of Myelin Basic Protein (MBP) was detected by Western Blotting. β – actin was used as a loading control, samples are from Pre-Frontal Cortex after six weeks of CPZ diet and LFMS treatment. (B) Relative MBP/ β – Actin ratio. Data is presented as means± SEM (n=5-6 per group). ****p<0.0001 vs. CTL, and #p<0.05 vs. CPZ.

Figure 3.14: Western blot of GFAP after 6 weeks of acute CPZ demyelination

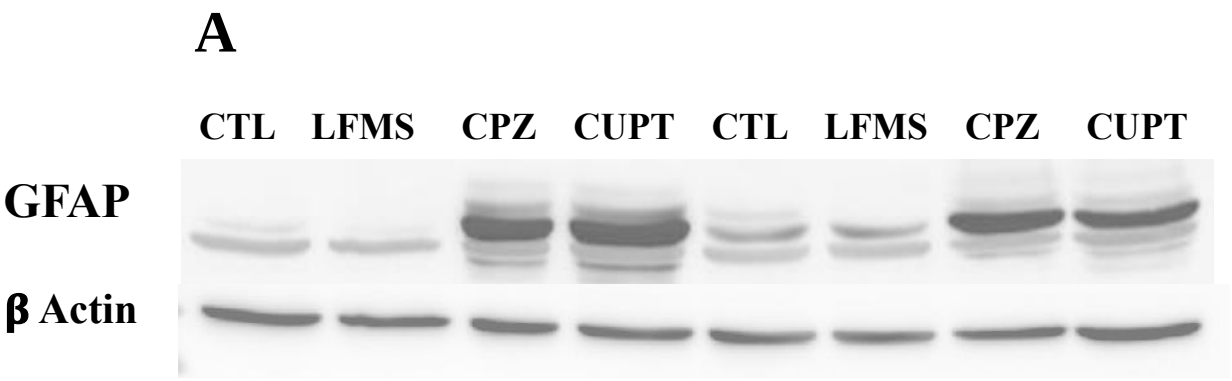


Figure 3.14: A) Glial Fibrillary Acidic Protein (GFAP) expression level detected by Western Blotting for prefrontal cortex samples after 6 weeks of CPZ diet and LFMS treatment, B) Relative GFAP/β-actin ratio, ***p<0.0005. Vs. CTL.

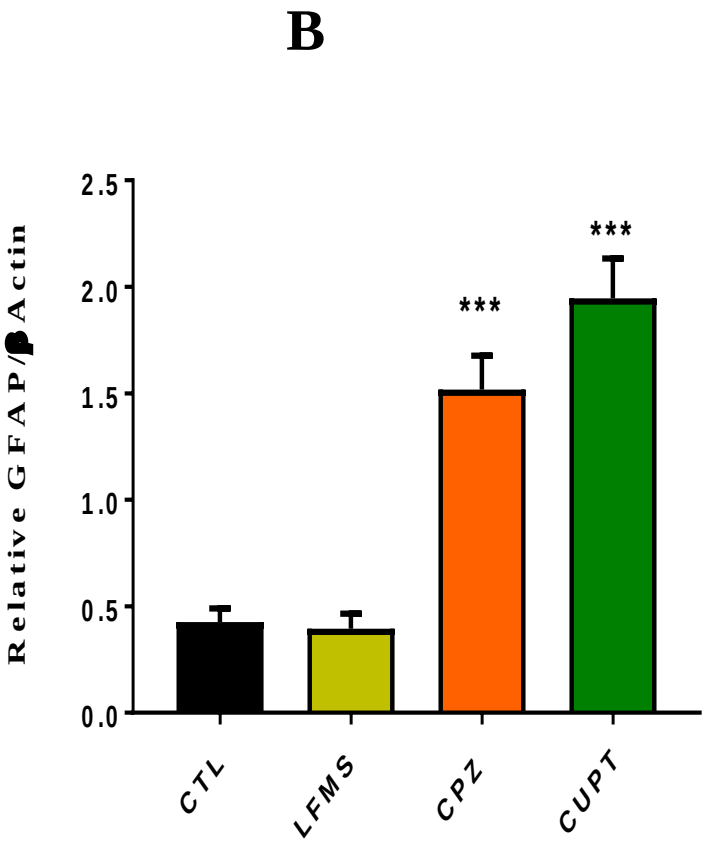


Figure 3.15: Western blot of TGF-β1 after 6 weeks of CPZ and LFMS in prefrontal cortex and hippocampus areas.

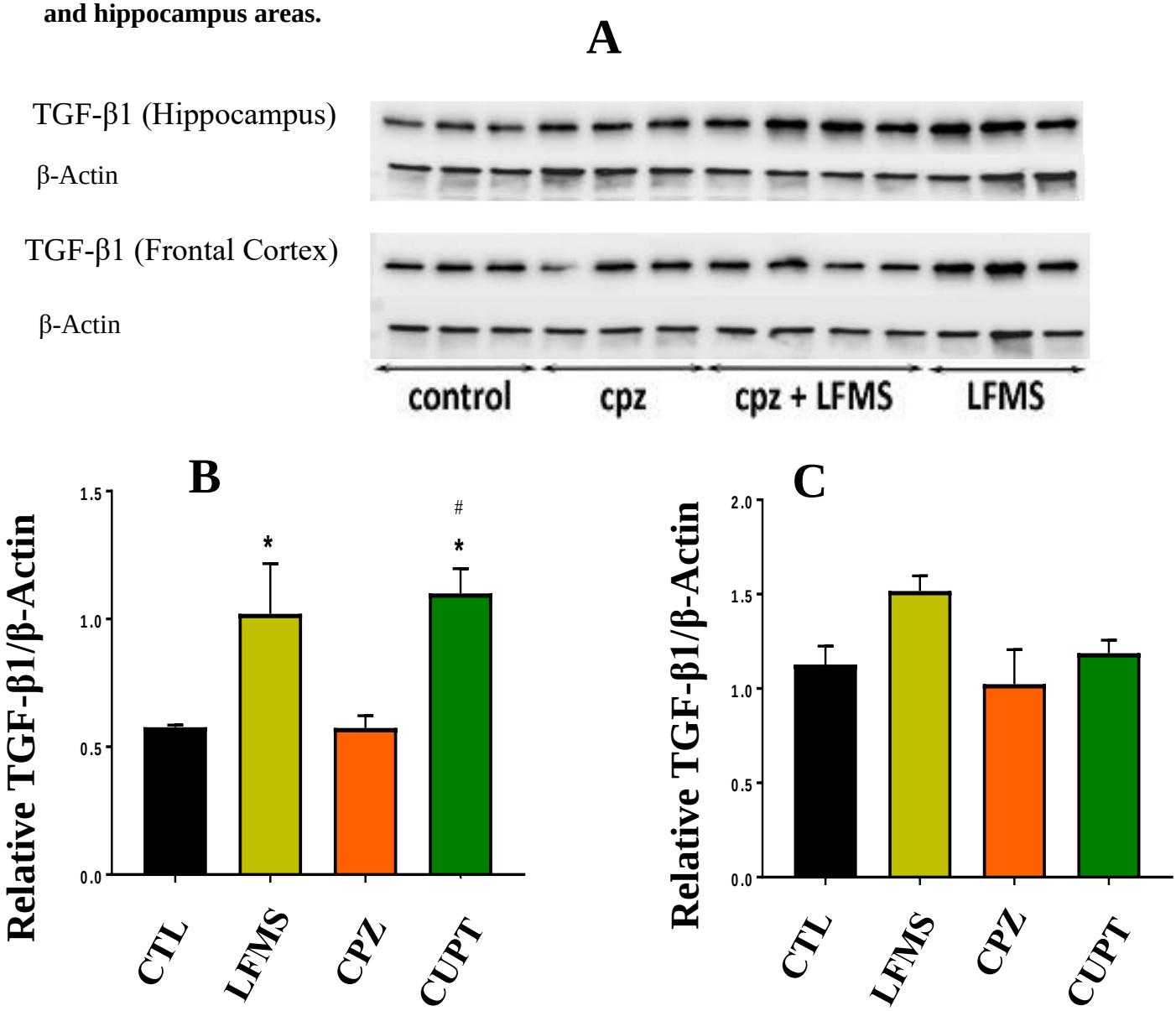


Figure 3.15: (A) Transforming Growth Factor Beta1 (TGF-β1) was detected by Western Blotting. β – actin was used as a loading control, samples are from Hippocampus of the animals after 6 weeks of CPZ model. (B) Relative TGF-β1/ β – actin ratio for samples from Hippocampus. Data is presented as means± SEM (n=3-4 per group). *p<0.005 vs. Sham Treatment with no CPZ, # p<0.005 vs. Sham Treatment on CPZ diet. (C) Relative TGF-β1/ β – actin ratio for samples from frontal cortex after completion of 6 weeks of CPZ model.

Figure 3.16: Pathology of MBP in frontal cortex after 3 weeks of CPZ diet and LFMS

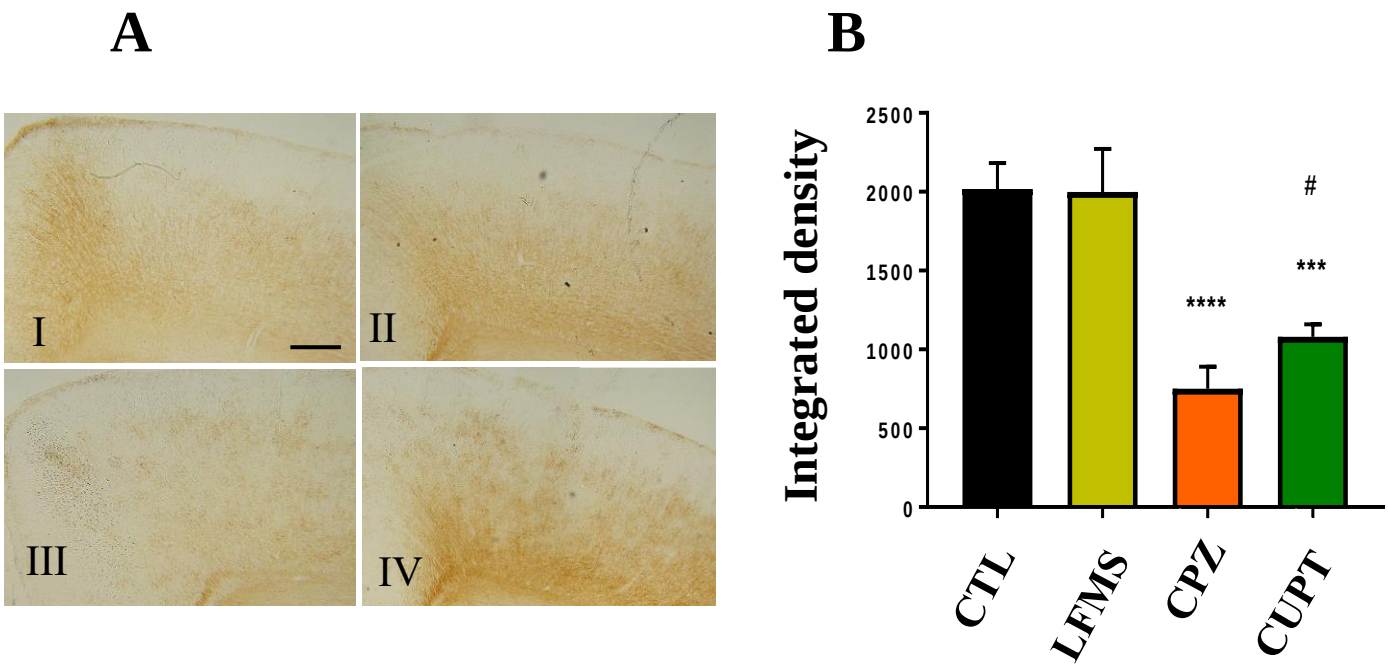


Figure 3.16: (A) Partial Illustration of Immunohistochemistry (IHC) for MBP in sections from Frontal Cortex after three weeks of CPZ diet [I: CTL, II: LFMS, III: CPZ, IV: CUPT], the scale bar represents 200µm. (B) Shows the quantification results of (IHC). Data is presented as means± SEM for Integrated density (n=4-8 per group), *** $p<0.001$ vs. CTL, ****$p<0.0001$ vs CTL. # $p<0.05$ vs. CPZ

Figure 3.17: Pathology of MBP in hippocampus after 3weeks of CPZ diet and LFMS

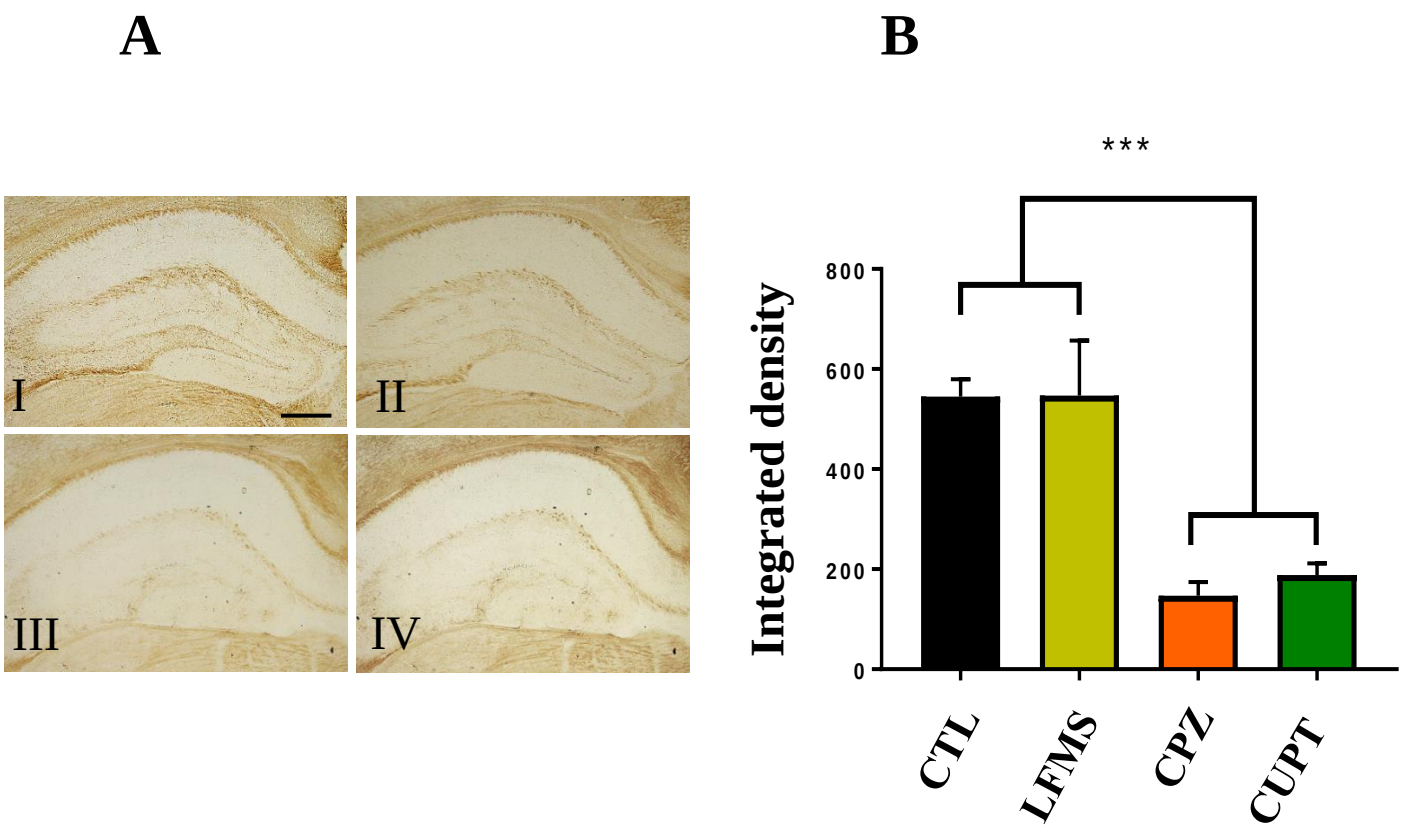


Figure 3.17: (A) Partial Illustration of Immunohistochemistry (IHC) for MBP in sections from Hippocampus after 3 weeks of CPZ diet [I: CTL, II: LFMS, III: CPZ, IV: CUPT], the scale bar represents 200µm. (B) Shows the quantification results of (IHC). Data is presented as means± SEM for Integrated density (n=4-6 per group), *** $p<0.0001$ vs. CTL.

Figure 3.18: Pathology of MBP in Frontal cortex after 6 weeks of CPZ and LFMS

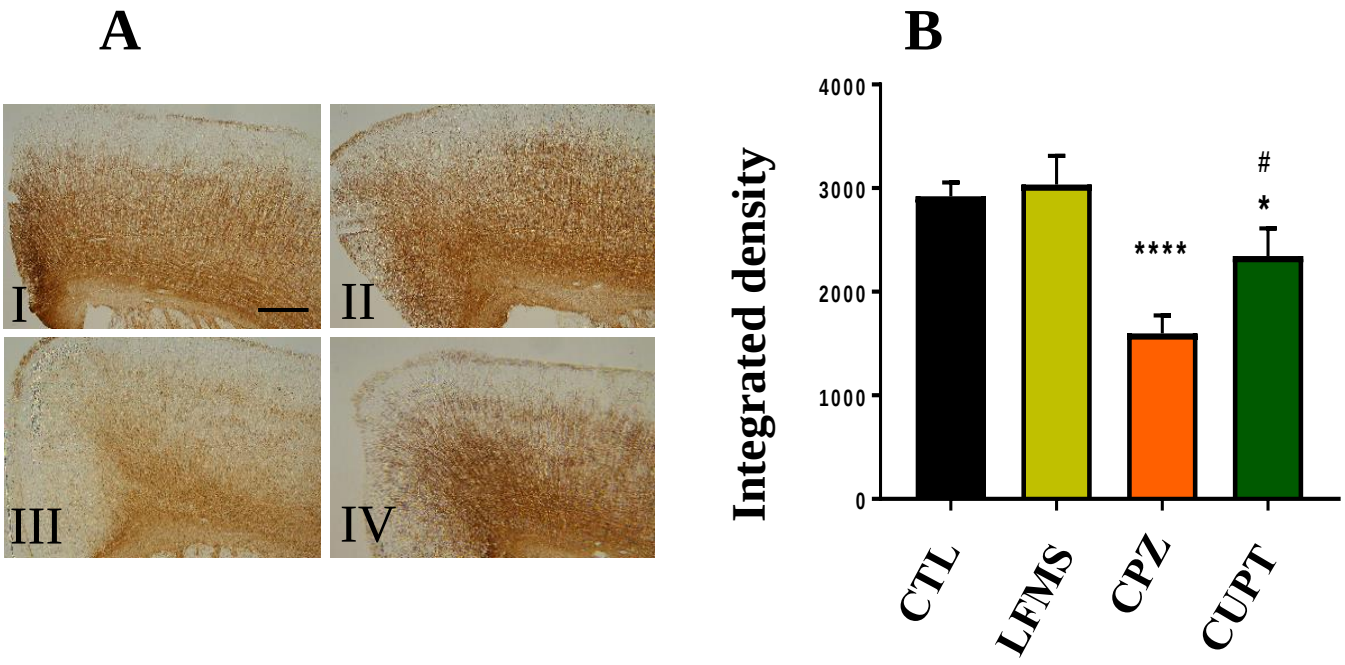


Figure 3.18: (A) Partial Illustration of Immunohistochemistry (IHC) for MBP in sections from Frontal Cortex after 6 weeks of experiment [I: CTL, II: LFMS, III: CPZ, IV: CUPT] the scale bar represents 200µm. (B) Shows the quantification results of (IHC). Data is presented as means± SEM for Integrated density (n=4-6 per group), **** $p<0.0001$ vs. CTL. *$p<0.05$ vs. CTL, # $p<0.5$ vs. CPZ

Figure 3.19: Pathology of MBP in hippocampus after 6 weeks of CPZ and LFMS

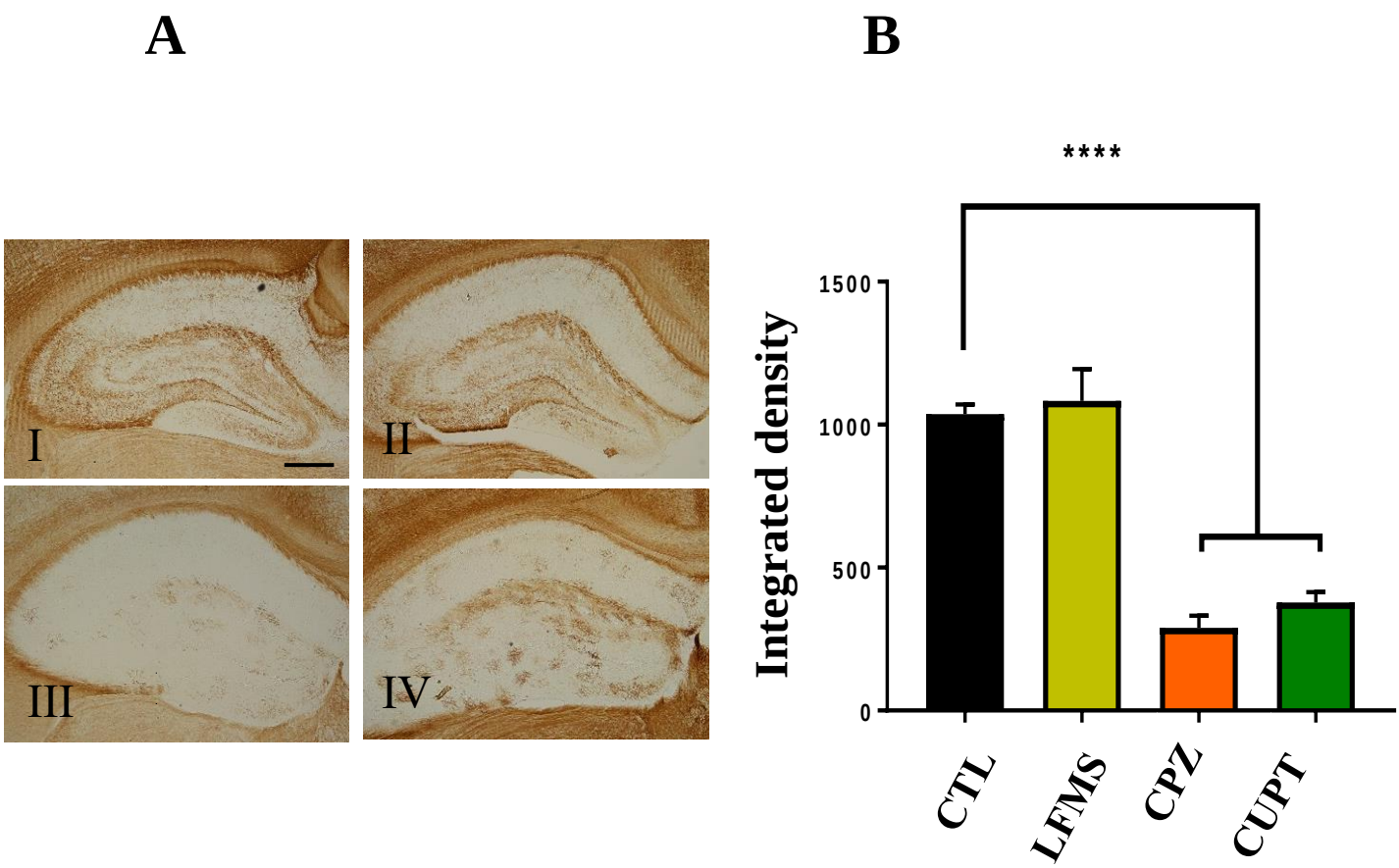


Figure 3.19: (A) Partial Illustration of Immunohistochemistry (IHC) for MBP in sections from Hippocampus after 6 weeks of experiment [I: CTL, II: LFMS, III: CPZ, IV: CUPT] the scale bar represents 200µm. (B) Shows the quantification results of (IHC). Data is presented as means± SEM for Integrated density (n=4-6 per group), **** $p<0.0001$ vs. CTL

Figure 3.20: Comparison of weight change among groups after 6 weeks of CPZ and LFMS

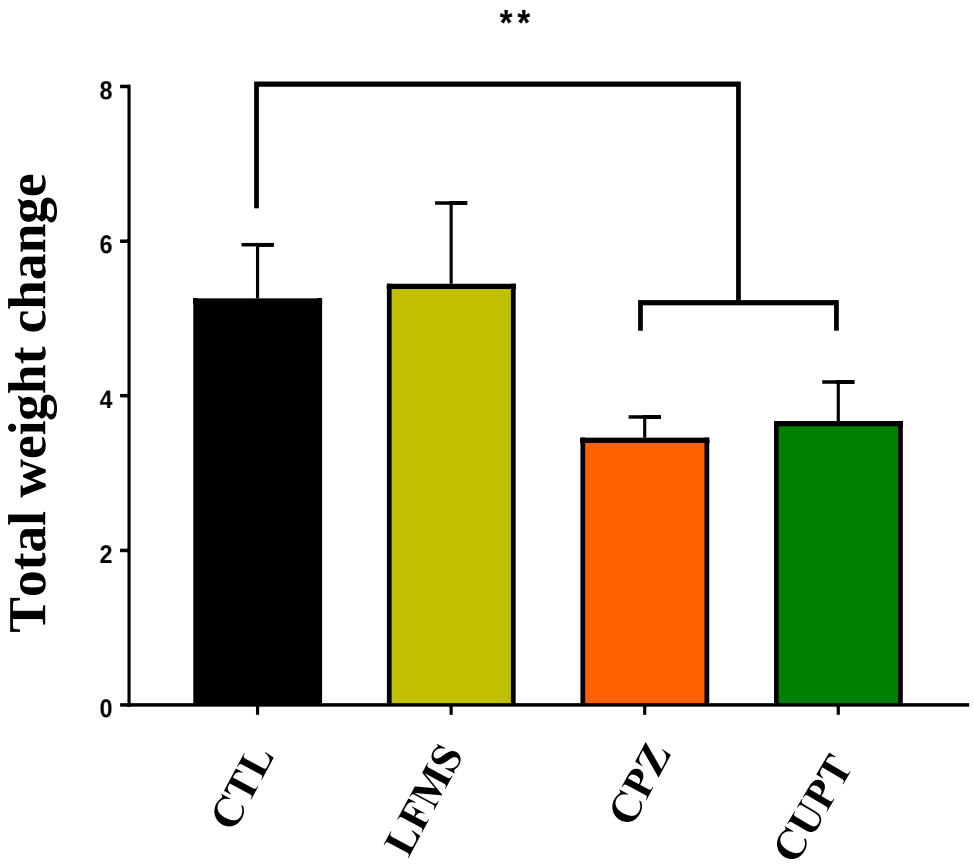


Figure 3.20: Comparison of weight change between groups at the end of 6 weeks of CPZ model. ** $p<0.005$ vs. CTL

Figure 3.21: weight change in the first four weeks of the experiment within groups

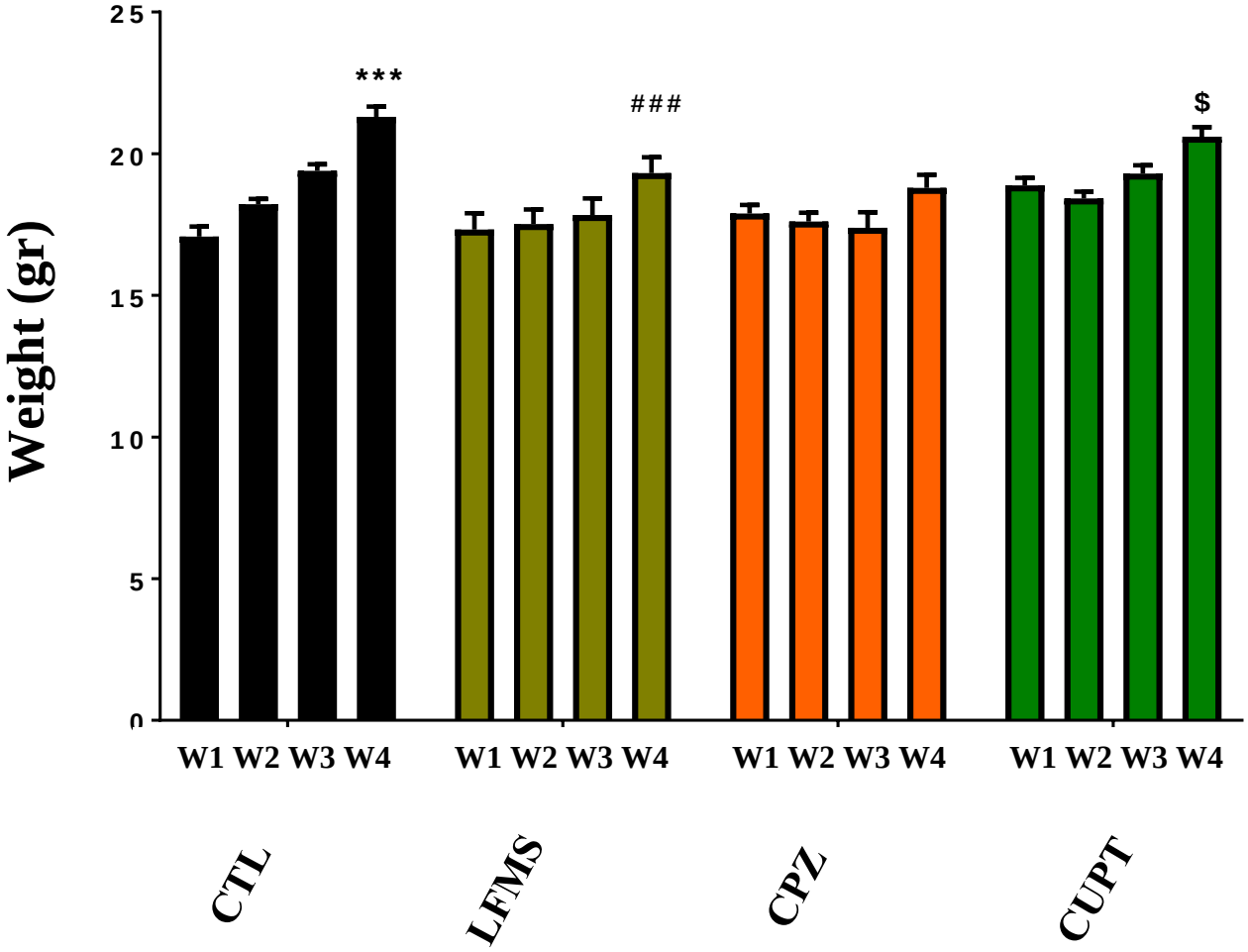


Figure 3.21: Comparison of the average weight of the groups from week 1 to week 4. Each bar from the same color represents the average weight at the end of a week of CPZ model. *** $p<0.005$ vs. lowest CTL weight, ### $p<0.005$ vs lowest LFMS weight, $ $p<0.05$ vs. lowest CUPT weight.

CHAPTER 3. DISCUSSION

OFT revealed no motor deficit, while LFMS helped with impulsivity

Unlike the experimental autoimmune encephalomyelitis (EAE) model of demyelination/MS, which is notorious for induction of motor deficit in mice, the CPZ model rarely causes problem with the motor system (Xiao et al., 2008; Yanbo Zhang et al., 2007; Y. Zhang et al., 2008). The paucity of motor impairment was one of the features of the CPZ model which encouraged us to employ it for studying cognitive status. Yet, since the possibility of developing motor deficit could not be completely ruled out (Mohamed et al., 2019), we decided to check the mice for motor function. This was done by measuring the distance that the animals travelled and the lines that they crossed during OFT and Y-maze (Li et al., 2014; Seibenhener & Wooten, 2015; Y. Zhang et al., 2012). These tests were performed at the beginning of the behavioral test battery both after 3 weeks and 6 weeks of CPZ diet. Distance travelled by the animal and line crossing are both indicators of motor function and mobility, although line crossing is the older, more crude way of measuring the mobility of the animal. Although we had the measures for the former from the software, we calculated the values for the latter as a way to back up the results of the distance travelled. Based on the results, we noticed little difference between the groups, neither for the first behavioral test battery nor during the second time. The immobility time and mean speed of the mice were also measured as additional values to compare the motor function of the animals. This made us confident that any differences in other parts of the behavioral tests are exclusively attributed to cognitive and emotional status of the animal in this specific study. Needless to say there are other well established tests to assess the motor coordination and balance in rodents, namely, rotarod test and beam crossing test; nevertheless,

the data that we have from OFT and Y-maze serve our purpose to compare the gross movements of our subjects and make sure that no group is limited in ability to move around.

It has previously been shown that mice under the effect of CPZ demonstrate significantly more travels to the center of the OFT when tested after 3 weeks and 6 weeks of the model (Franco-Pons, Torrente, Colomina, & Vilella, 2007) and this phenomenon has been attributed to developing impulsive behavior in CPZ model. The reason behind the mice being incapable of controlling their impulses has been described as a result of white matter deficit (Silveri et al., 2006). Our mice showed the exact pattern after 3 weeks of CPZ diet. However, when tested after 3 more weeks, the +CPZ/+LFMS group showed a decline in the average of their central time, a behavior pattern closer to the CTL group. We still have no explanations as to why the CPZ/+LFMS mice showed significantly more exploratory behavior during the first OFT. Further investigation, especially more anxiety oriented tests like elevated plus maze could be helpful to shed more light on our findings.

Acute CPZ model of demyelination induces short term memory loss

Y-maze results confirmed the ability of CPZ to induce deficit in short term memory (interchangeably used as working memory) (Cowan, 2008), which was depicted as a lower percentage of spontaneous alternations among the mice who were on CPZ diet compared to the **CTL** (Cowan, 2008). In other words, CPZ caused the mice to have more trouble remembering which arm of the Y-maze they visited last. Animals that received CPZ diet but no LFMS (**+CPZ/- LFMS**) showed more significant short term memory loss after completing 3 weeks as opposed to 6 weeks of CPZ diet ($P<0.0001$ after 3weeks compared to $P<0.05$ after 6weeks). It was previously shown that the features of cognitive impairment, namely the severity of short

term memory deficit, is alleviated once the CPZ diet is cut at the end of the acute model of CPZ and 2-3 weeks have passed (Xu, Yang, Rose, & Li, 2011). It has been shown that spontaneous remyelination starts after the third week of CPZ diet and becomes prominent during the sixth week (Praet et al., 2014). Our results are in line with these findings, considering the fact that severity of short term memory loss in this model is significantly higher compared to **CTL** when inflammation and demyelination are documented to be at their peak. This memory deficit is present but less significant when the animals were tested at the end of the standard 6 weeks of acute demyelination of CPZ.

LFMS has protective effect on C57/bl6 mice against short term memory loss

It is well documented that week 4 and 5 of the CPZ model is the peak time of inflammation/demyelination (G. K. Matsushima & P. Morell, 2001; Praet et al., 2014; Skripuletz, Gudi, Hackstette, & Stangel, 2011; Yu et al., 2017). It is also essential to consider the fact that remyelination starts at the very basic level during the same time but it takes about two more weeks for remyelination to become prominent (Praet et al., 2014). From this moment, the two opposing phenomena enter a combat in which demyelination predominates unless the CPZ is cut from the diet. It is only logical to infer that any intervention that could alleviate the symptoms caused by demyelination, while the mice are still on CPZ diet, could be considered to have potential for protective effects against demyelination. In our study, while both groups of mice on CPZ diet showed signs of short term memory loss after 3 weeks of the experiment, the severity of this deficit was less significant in those who had received LFMS treatment; thus, LFMS appears to have had protective effects against the memory loss caused by CPZ. The timing of the first behavioral test battery was concurrent with the period which resembles the

highest inflammation and demyelination and far from any prominent touch of remyelination; thus, it sounds plausible to assume that LFMS conveyed those protective effects partially, if not entirely, by a different mechanism other than enhanced remyelination. A possible rationale is altered inflammatory status of the brain tissue; therefore, future studies of inflammatory elements like M1 and M2 microglia and/or inflammatory cytokines including TNF-α and Interferon- γ might be a good start to achieve a more conclusive set of results to explain the mechanism behind the effects of LFMS.

When the subjects were tested after 3 more weeks of experiment the +CPZ/+LFMS group did not show any sign of significant memory loss, while those animals who did not receive LFMS were still struggling with CI. At this point the former group showed even significantly better results than the latter. Since week 6 is marked as the time that remyelination occurs, and our behavioral test was performed a week later, the significant difference between the two CPZ fed groups might very well be due to enhanced remyelination. Besides, what we assess through a one trial form of Y-maze, is by nature independent from the memories that the animal carries; therefore, a battery of cognitive state after the end of week 3 cannot be considered as an advantage for a later test of the same nature (Voikar, Vasar, & Rauvala, 2004), meaning that, better results among LFMS treated mice after week 6 should be regarded as a sign of continuous effective treatment.

LFMS had protective effect against depressive-like symptoms in classic FST model

FST, as a tool to predict the probability of anti-depressant treatment success, is done merely once for the mice who are naïve to the test [105]. To the best of our knowledge, there has

been only one study which was carried out by our lab and checked the depressive-like symptoms in the CPZ model using FST (Y. Zhang et al., 2019). In the previous study, FST was carried out during the fifth week of the study, while in the current study we did the FST after the completion of the third week. While no significant differences were found between groups in the previous study, here we found that the **+CPZ/-LFMS** group demonstrated the classical sign of despair, that is to say a significantly longer *total immobility time* compared to **CTL.** At the same time, **+CPZ/+LFMS** mice were not significantly different from the **CTL** group and were significantly different from **+CPZ/-LFMS.** In other words, the mice who received CPZ and were treated with LFMS dealt with a desperate situation the same way that normal mice do and spent the same portion of their time on actively trying to escape from the unescapable situation. In terms of the *latency to the first immobility*, **+CPZ/-LFMS** group were also abnormal**.** It took these mice a significantly shorter time to show the signs of despair, which in this scenario, is indicated by the mouse giving up trying to escape. At the same time the CPZ fed mice who had received LFMS, were not significantly different from their normal peers, and coped significantly better with the stressful situation than those who were not treated. Choosing further time points to do the FST during the CPZ model might be helpful to assess the effect of LFMS in different conditions than the ones we studied. Furthermore, other behavioral tests such as "Sucrose preference test", which are designed to target and evaluate depressive-like symptoms in the rodents could be useful to generate further supporting data.

Serial FSTs as a tool for evaluating cognitive impairment

FST was originally designed as a model to induce depressive-like symptoms and test the efficacy of antidepressant interventions. Yet, two more categories of data interpretation have

long been known for FST. In the mid 1980s, the papers that described using FST as a method to assess depressive-like behavior were equal in number to the papers that described using FST as a tool to evaluate other aspects of behavior like memory and learning (de Kloet & Molendijk, 2016). Recent findings have provided us with additional insight regarding the use of serial FSTs in cognitive assessment (Mul et al., 2016). In brief, it has been documented that even a single exposure to FST increases the *total immobility time* during a next day FST and if retesting continues, the immobility time will also increase accordingly. Some studies considered this increased immobility as a sign of increased despair (Alcaro, Cabib, Ventura, & Puglisi-Allegra, 2002). These studies explained this higher immobility time with "learned helplessness" and even attributed it to a higher vulnerability to depressive-like symptoms. Nevertheless, we tend to side with the other perspective that perceives the increased immobility time, in repeated FST, as a part of the animals' *coping mechanism* (de Kloet & Molendijk, 2016) (Han & Nestler, 2017) to a stressful situation, an adaptive and learning behavior. In other words this is an evolutionary mechanism leading to less stressed behavior, energy expenditure and eventually better chance of survival. Coping mechanisms are either active or passive and decreased escape attempts in this case is documented to be part of extinction-associate inhibitory learning which involves different parts of the brain, namely, prefrontal cortex and hippocampus (de Kloet & Molendijk, 2016). It is also a complex of cognitive elements like short term and long term memory, information processing and executive function.

In addition to total immobility time, the period between the start of the test and the moment that the animal becomes immobile for the first time is also measured and used as a quite sensitive tool to check the level of despair in standard FST. We also checked this measure to observe the possible changes in our animals. In the standard FST, the sooner the first immobility

happens, the more severe depressive-like symptoms of the mice are. Although to our knowledge this parameter has not been checked in previous serial FSTs, we expected it to be affected by the memory of the original stressful situation (first time FST).

Another term which might be appropriate to mention is “habituation”. Habituation is termed as “the simplest form of learning” and is defined as “when repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level (Rankin et al., 2009). This change may include decreases in frequency and/or magnitude of the response.” In our case, re-exposing the mouse to a stimulus (FST) leads to progressive decline in the time that the mouse spends on actively trying to escape, which is manifested as increased immobility time.

Here we designed a test battery of serial FSTs. The first two FSTs were done after the completion of the third week of the experiment 24 hrs apart from each other. This set of FST aimed to test the long term memory when inflammation and demyelination are at their peak. The next two FSTs were run after the completion of the 6^{th} week of the experiment and were 24 hrs apart from each other. The second pair of FSTs were used to evaluate the effects of CPZ and LFMS on long term memory retention right after the standard time for the acute CPZ demyelination had elapsed. It has been documented that a normal mouse brain is capable of retaining the memory of a stressful event even after ten weeks from a test battery (Nishida et al., 1997) and use that memory to act accordingly. Moreover, the passive coping mechanism in serial FSTs is documented to last for 4 weeks after the last FST (de Kloet & Molendijk, 2016); thus, a gap of 3 weeks between the two sets of FST sounds practical enough to serve our purpose.

Impaired coping mechanism in CPZ model implicates cognitive deficit

As expected, we noticed that the CTL mice demonstrated a longer immobility time during the second FST (de Kloet & Molendijk, 2016) (Alcaro et al., 2002) (Voikar et al., 2004). Treating the mice on normal diet with LFMS had similar results except for their increase in immobility time was significantly higher than the CTL group, which does not raise much concern for three reasons. First, previous studies showed that this increase in the first retest could be significant in CTL groups as well (Mul et al., 2016); therefore, our different results could be attributed to possible varieties in performance details and in the mice's behavior. Second, difference in the behavioral change is not different in trend but in amplitude which could be negligible if it is congruent with other elements of the animals behavior. Third, if the same results are obtained in future studies and is supported by other extensive behavioral tests, it could be regarded as the potential of LFMS to enhance habituation in normal mice.

No coping failure in LFMS treated mice

Those animals who were on CPZ diet, but also received LFMS, did not show any increase in their total immobility time compared to their first FST which statistically was not significantly different from the CTL group. On the contrary, CPZ diet without LFMS apparently disrupted the animals' coping process. This increased activity time, compared to what was expected as in our CTL, could be described as a sign of failure to cope with the stressful situation or habituation; thus, it could be crudely marked as a deficit in learning, memory, information processing and executive function because all these elements are required for a healthy coping mechanism to happen. Since this decline in the total immobility time (in other words time that the mice spent on actively trying to escape) cannot be taken as a sign of alleviated depressive

symptoms (Mul et al., 2016)(de Kloet & Molendijk, 2016), we tend to interpret it as a possible sign of increased anxiety, in other words, a manifestation of panic.

When the animals in **+CPZ/-LFMS** group were re-tested after 3 more weeks of CPZ diet, their total immobility time was even significantly less than at the previous testing time. This could be the result of an impaired cognition at the time accumulated with the paucity of proper memories in order to help them with coping. In contrast, our **CTL** mice showed significantly higher immobility time as expected. The same value was not significant for the normal diet mice who received LFMS. At this point we have no explanation for this difference between our two normal diet groups. The results from the last FST did not show any significant changes in any of the groups except for **+CPZ/+LFMS**. This is where the added FST results differ in nature with Y-maze as a mouse requires the memories from the previous tests if they want to manifest a proper coping mechanism, and unlike Y-maze the results from the secondary tests cannot be interpreted independently (Voikar et al., 2004). Based on this statement we could infer that since LFMS was given from day one of the experiment and our results show that it was successful in preventing the **+CPZ/+LFMS** mice from having a dramatic deficit after week 3, part of the significantly promising results for these treated mice at the very last round of FST could be attributed to their better preserved memory in early stages of the experiment.

Interpretation of the figures for the latency to the first immobility episode leads to the same conclusions. During the first retest, only the **+CPZ/-LFMS** group had a significantly higher value compared to the **CTL**. That is to say, only those mice who were on CPZ but did not receive LFMS failed to cope with the stress of the second test. Based on the results of the last round of FST, it took them a total of four exposures to finally demonstrate a coping response that

other groups showed after one exposure, while LFMS treatment was successful in preventing this scenario in the first place for the treatment group.

LFMS protected the frontal cortex from demyelination but not the hippocampus

Our IHC results demonstrate a trend in demyelination in accordance to previous similar studies (Steelman Andrew J. et. al., 2012). In order to answer our objective questions, we started the first set of data gathering after 3 weeks of CPZ diet, and the brains were harvested after the behavioral test batteries were done. This provided us with the opportunity to check the animals' cognition and emotional state at the peak of demyelination/inflammation as the IHC results indicate. While both CPZ fed groups were severely demyelinated, the LFMS treated mice showed a less significant demyelination status in their frontal cortex compared to the CTL. At the same time, LFMS did not show any protective effects on the hippocampus, an observation which cannot be yet explained. A better understanding about the mechanism behind the LFMS effect on the brain might be helpful in terms of finding a rationale for this result. So far, we know that in CPZ model, the hippocampus area has a slower remyelination rate compared to the other areas of the brain (Steelman Andrew J. et. al., 2012). Yet, further investigation is needed to determine whether LFMS has the ability to accelerate the remyelination in the hippocampus. Having a comprehensive comparison between the two groups with CPZ diet in more time points during the remyelination phase might be helpful. Based on previous studies we know that although remyelination starts during the third week of the experiment, there are no obvious behavioral effects at this point (Praet et al., 2014). Therefore, it is not unreasonable to assume that LFMS had a protective effect against demyelination rather than augmenting the

remyelination process. However, in order to make such a claim it is necessary to compare the remyelination process at the same timeline, something that is yet to be done with specific methods that evaluate remyelination, namely using the 5 bromodeoxyurine (BrdU assay).

After completing 6 weeks of CPZ diet the brain sections showed a milder demyelination. At this point, the severity of demyelination in frontal cortex was even less in the +CPZ/+LFMS group compared to the untreated ones to the point that it was statistically significant. We used Western blotting in order to double check this significant change and the results supported the IHC findings. At the same time, LFMS seemed to have been unable to make any changes in the hippocampus area.

Further investigation is required to give us a clearer picture of myelin status after LFMS treatment in various areas of the brain in different time points. Since extensive remyelination has been shown to occur by week 6 of the CPZ model (Praet et al., 2014), a comparison of the remyelination state between the two CPZ fed groups, using the 5 bromodeoxyurine (BrdU assay), could be helpful in researching the possibility of LFMS being capable of enhancing remyelination in CPZ model.

LFMS treatment increased TGF-β1 in the brain tissue

We chose to check TGF- β1 in the mice brains as a possible mechanism for several reasons. First, TGF- β1 has been documented to be a major role player in regulating inflammation in the human body (Xie et al., 2018) and inflammation plays an enormous role in the pathology of demyelination in MS and CPZ model. Second, we noticed an overall improvement in myelination status in our treatment groups and TGF- β1 has been shown to be

involved in regulating myelination (Xie et al., 2018). Finally, TGF- β1 is increasingly being considered as a possible option in MS treatment (Xie et al., 2018)(Nishida et al., 1997) for reasons ranging from its general regulatory role in autoimmune phenomena to its more specific ability to affect microglia polarization.

Our data from Western blot assay shows that LFMS treated groups, regardless of their diet, had significantly increased TGF- β1 in their frontal cortex and hippocampus. Comparison of TGF- β1expression between groups in different areas show that while the trend of change is the same, the results were only significant in the hippocampus. Aside from the possibility of different responses to the same treatment among different areas of the brain, this finding could be due to the fact that at the time of our sample collection (after 6 weeks of CPZ followed by one more week for behavioral tests) the frontal cortex showed a significantly alleviated myelination state, while the hippocampus was still struggling to recover. This could be caused by different rates of expression of TGF-β1 in different areas of the brain (Nishida et al., 1997). Further investigations of these findings by both repeating the same method and employment of even more comprehensive techniques are needed to reach more conclusive results, but we find this data worthy enough to be reported. Further investigation is needed to locate the exact brain areas that bear TGF- β1 while CPZ and LFMS are administered for the animals e.g. Immunofluorescence and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Moreover, the mechanism(s) by which LFMS increases TGF- β1 might be studied by checking the elements in TGF- β1 signaling pathway, namely SMAD protein (Araujo Ana P.B. et. al., 2016).

CHAPTER 4. CONCLUSION

Psychiatric conditions in the course of MS are not being addressed as they should be. We suggest LFMS as a novel intervention to deal with these symptoms. Availability and affordability are the key positive aspects of this intervention. We designed an experiment to study the effects of LFMS on cognitive symptoms and depressive–like behavior of C57/bl6 mice in a CPZ model of demyelination/MS. We used various behavioral tests and lab techniques to tackle our objectives. Our results from the behavioral tests, immunohistochemistry and Western blotting supports our hypothesis. We used serial FST to assess the long-term memory and coping mechanism of the mice. To our knowledge this is the first time that the effects of CPZ on cognition have been studied with this test battery. Our data shows that CPZ induces a similar pattern of learning deficit as MS in C57/bl6 mice and LFMS showed potential protective effects against this cognitive impairment. LFMS was also revealed to be helpful in protecting the mice against deficit in short term memory (working memory). The antidepressant effect of LFMS has also proven to be a promising one. Our findings in behavioral tests are supported by pathological study of the mice's brains and biochemical quantification of myelin basic protein in the frontal cortex. However, LFMS fails to show similar ameliorating effects in the hippocampus area, a finding contradictory to some previous studies.

We studied the general presence of TGF- β1 in our subjects' brains, which revealed a significant increase in LFMS treated groups. As consistent as the latter finding is with our other results, we strongly encourage further more comprehensive assessment of TGF- β1 in LFMS related studies. If a positive conclusive result is achieved in this matter, the translational impacts could be greatly beneficial as TGF- β1 is increasingly being considered as an option in treating

MS. Yet some caution is warranted. First, TGF- β1 as a pharmaceutical product is not the cheapest option. Second, administering TGF- β1 as a medication comes with the routine risk of allergy which in turn adds to the overall risk of administration. Finally, administered TGF- β1 needs to pass the Brain Blood Barrier (BBB) in order to reach the brain as the target organ. If LFMS has the ability to increase TGF- β1 either by increased production or any other facilitating pathway, the benefits of this treatment should be assessed in clinical studies in order to get a better picture of how LFMS could be used to help MS patients with their imminent risk of cognitive loss.

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