Title: Intermittent Theta Burst Stimulation Mitigates Depressive-Like Behavior in Rats Subjected to Maternal Separation

Running Title: Magnetic Stimulation Mitigates Depression

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Abstract

Early life stress, such as maternal separation (MS), can lead to serious mental health problems such as depressive-like behavior later in life. Intermittent theta burst stimulation (iTBS) is a transcranial magnetic stimulation (TMS) protocol. In this study, we sought to determine the effectiveness of iTBS protocol on depressive-like behavior and cognitive impairment induced by MS in male rats because the therapeutic effect of this protocol on this model remains unknown.

To induce depression, we used the MS method from postnatal day (PND) 2 until PND 20 for 4 hours each day. At PND 30, the iTBS treatment began and continued for 10 consecutive days. At PND 40, behavioral tests (Barnes maze, open field test, elevated plus maze and forced swim test) were conducted, and the rats were sacrificed on day 50, with brain tissue removed for biochemical analysis (oxidative stress, TNFα, BDNF and BACE1).

The results showed that iTBS significantly improved spatial memory impaired by MS in the Barnes maze and significantly reduced anxiety-like behavior in the elevated plus maze and depressive-like behavior in the forced swim test. Biochemical analysis showed that iTBS significantly reduced oxidative stress (SOD, ROS, MDA, CAT, and GSH), inflammation (TNFα) and BACE1, and increased BDNF in the hippocampus.

Taken together, our study suggests that the iTBS protocol may have therapeutic effects on depressive-like behavior and cognitive impairment induced by MS.

Keywords: Intermittent theta burst stimulation, Maternal separation, Depression, Oxidative stress

1 Introduction

Major depressive disorder (MDD) is a prevalent disorder that can greatly affect daily functioning and severely impair quality of life (De Risio et al., 2020). One important factor that may cause psychiatric disorders, such as depression, is early life stress (Murgatroyd et al., 2015). It can lead to behavioral disorders that continue into adulthood, such as depression (F. Wang et al., 2020). Evidence suggests that early life maternal separation (MS) may have an adverse effect on brain development and lead to serious mental problems such as depression and autistic-like behavior (Bian et al., 2015; Mansouri et al., 2021; Zhou et al., 2020). Moreover, MS has been linked to cognitive deficits later in life (Hao et al., 2025; Suman et al., 2025). These findings highlight the need to understand how early stress alters specific brain regions, particularly the hippocampus, which is critically involved in mood regulation and memory.

The hippocampus is an important region of the brain involved in stress-related depression (Zhou et al., 2020). Studies report that depression is associated with reduced plasticity and decreased levels of brain-derived neurotrophic factor (BDNF) in areas such as the hippocampus (Ye et al., 2011). Early life stress, such as MS, can also impact memory performance (Diehl et al., 2012). In addition to behavioral changes, MS induces oxidative stress and neuroinflammation in the hippocampus. It can increase anxiety and decrease catalase (CAT) activity in the hippocampus (Malcon et al., 2020). Another study showed that MDA and nitric oxide increase and antioxidant levels decrease in the hippocampus after maternal separation in mice (Rostami-Faradonbeh et al., 2024). Studies also report increased inflammation in this area with prolonged MS (Zhou et al., 2020). TNFα expression was reported to increase in the hippocampus in another study (Farzan et al., 2023). These molecular disturbances are relevant to depressive-like mechanisms. For example, Beta-secretase 1 (BACE1) has been shown to be associated with cognitive impairment (Cheng et al., 2014), and oxidative stress is an important factor that can influence BACE1 activity in the hippocampus (Mouton-Liger et al., 2012). Given the

combined behavioral, inflammatory, and oxidative alterations, MS provides a suitable model for testing therapeutic interventions with fewer side effects.

Transcranial magnetic stimulation (TMS) is a non-invasive method for modifying brain activity using magnetic pulses (Shirota & Ugawa, 2024). Theta burst stimulation (TBS) is a specific TMS protocol (Lee et al., 2021). There are two commonly used protocols of TBS in research: intermittent TBS (iTBS), which is believed to facilitate neural transmission, and continuous TBS (cTBS), which suppresses brain excitability (Vékony et al., 2018). iTBS, consisting of 3x50 Hz pulses repeated at 5 Hz, has been reported to have similar antidepressant effects compared to the traditional high-frequency rTMS protocol (Lee et al., 2021). Beyond mood regulation, iTBS has shown cognitive-enhancing effects (Pabst et al., 2022), and it has also been reported that iTBS has an antioxidant effect and increases BDNF in the hippocampus and other regions in a rat model of Alzheimer's-like disease (J. B. Stanojevic et al., 2023). In another study of autism induced by maternal separation, low-frequency rTMS applied for 14 days attenuated behavioral symptoms by regulating GABA transmission in the hippocampus (Tan et al., 2018).

While previous studies have explored the effects of low frequency rTMS in autism induced by MS (Tan et al., 2018), the potential therapeutic role of iTBS in depressive-like behaviors induced by MS remains unclear. Based on this knowledge, we hypothesize that the iTBS protocol may improve depressive-like behaviors induced by MS in rats.

2 Material and methods

2.1 Experimental procedure

This study followed ethical guidelines and was approved by the ethics committee of Baqiyatallah University of Medical Sciences (IR.BMSU.AEC.1402.030). Male and female Wistar rats were housed together for mating. The male rat pups were separated from their mother and each other from postnatal day (PND) 2 to PND 20 for 4 hours daily, from 8 am to 12 pm, and others were handled daily (R. Wang et al., 2020). After PND 21, male rats were assigned into four groups (N=8): Sham, iTBS+H (iTBS + Healthy rat), MS, and MS+iTBS. All rats were maintained in standard animal housing conditions

(standard nesting materials, 12:12 h light/dark cycle) at 22 ± 1°C and 45 ± 3% humidity, with free access to food and water (Fig. 1).

2.2 iTBS treatment

iTBS treatment was performed in accordance with reference studies on another neurodevelopmental model (Rittweger et al., 2021). In this study, Super Rapid2 Magstim device equipped with an air-cooled, figure-eight-shaped coil (D70 air film coil) was used. Rats received 3 blocks of iTBS and 15-minute intervals between blocks (frequency: 50 Hz, number of pulses: 3, number of bursts: 10, cycle time: 10 s, number of cycles: 20 and total number of pulses: 600).

The stimulation intensity was set at 100% of the motor threshold, based on previous experiments with 5 healthy rats for forelimb movement (Tan et al., 2018), and set to 50% of the maximum device output. For restraint, in line with a previous study (Afshari et al., 2024), rats were introduced to the restraint method and device sound for one week before the actual treatment began (they were assigned to groups at PND 21). The rats were immobilized using medical gloves and a self-adhesive elastic wrap to minimize their movement during the application of the TMS coil, with the coil handle aligned perpendicularly to the rat's body (the center of the coil was positioned at the midpoint between the eye and the ear). All experiments were conducted under environmentally controlled conditions aimed at minimizing stress. In the sham group, the coil was placed upside down and positioned at a sufficient distance from the rats' head to prevent any actual stimulation, while replicating the auditory output of the coil. The treatment protocol started on PND 30 (Tan et al., 2018) and continued for 10 consecutive days.

2.3 Barnes maze test

The Barnes maze test was conducted to measure spatial memory, following previously published methods (Zappa Villar et al., 2018). The test includes three stages: habituation, training, and probe. During the habituation stage, rats were given enough time to explore the maze. In the training stage, rats learned to find the escape box and were expected to memorize its location. In the probe stage, the escape box was removed, and the rats' spatial memory was assessed. Several parameters were measured

during the test: latency: The overall time the rats needed to find the previous escape hole location for the first time during the probe test. number of errors: The number of explorations of the holes throughout the maze until the escape hole was found. strategy: The strategy the rats used to find the escape hole during the probe test. This was scored as follows: Score 1: Spatial (the rats directly went toward the escape hole). Score 2: Serial (the rats explored a series of holes next to each other until they found the escape hole). Score 3: Random (the rats had no specific pattern for finding the escape hole).

2.4 Open field test

In accordance with a previously published method (Mansouri et al., 2021), the time spent in the center of the apparatus over 15 minutes was assessed (40 × 40 × 40 cm).

2.5 Elevated plus maze (EPM) test

In accordance with a previous study, we used the EPM task to assess the anxiety levels of the rats (Servadio et al., 2016). One behavior was recorded: the total time spent in the open arms of the EPM for 5 min in total.

2.6 Forced swim test (FST)

To assess depression, the FST was employed. A cylindrical container was half-filled with water (25 ± 1°C). The test included a 2-minute acclimation period followed by a 5-minute test period (Hu et al., 2017). The duration of immobility in the water was recorded.

2.7 Biochemical analysis

After the behavioral tests, on PND 50, the rats were sacrificed following anesthesia (ketamine/xylazine). The hippocampal tissue was homogenized and stored in a -80°C freezer for further analysis. For oxidative stress analysis, the following methods were used: Reactive oxygen species (ROS) were measured using the dichlorofluorescein diacetate (DCF-DA) reaction. Based on the protocol, 100 µl of prepared supernatant and 10 µl of DCF-DA were added together and incubated in 37 °C for 30 minutes (excitation: 488 nm, emission: 525 nm) (Nazari-Serenjeh et al., 2024). Malondialdehyde (MDA) was determined using the TBARS (thiobarbituric acid reactive substances) assay. After mixing with supernatant and incubating for 80 minutes in 90 °C following cooling and centrifugation for 10 minutes at 1000 g (absorbance: 532 nm) (Alizadeh Makvandi et al., 2021).

Catalase (CAT) activity was measured by its ability to decompose hydrogen peroxide into water. For CAT assay, 20 µl supernatant was added to the buffer (100 µl) and diluted substrate (20 µl), incubated for 20 minutes, then potassium hydroxide (30 µl) was added to stop the reaction; after 5 minutes, potassium periodate was added (absorbance: 540 nm) (Nazari-Serenjeh et al., 2024). Glutathione (GSH) was assessed following the method detailed in previous literature by adding supernatant to DTNB (absorbance: 412 nm) (Alizadeh Makvandi et al., 2021). Superoxide Dismutase (SOD) activity was measured using the reaction of the supernatant with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8) at 37°C, incubated for 40 minutes, followed by addition of nitroblue tetrazolium and reading at 550 nm (Alizadeh Makvandi et al., 2021).

Additionally, Tumor Necrosis Factor Alpha (TNFα) levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with commercial kits from Bio-Techne, USA. Brain-Derived Neurotrophic Factor (BDNF) content in the hippocampus was assessed using a BDNF ELISA kit (RAB1138, Sigma, USA). BACE1 activity was evaluated by measuring the hydrolysis of DL-BAPNA per hour (ΔA/h) (Ogunsuyi et al., 2020)and protein content in the hippocampus supernatant was measured using the BCA (bicinchoninic acid) method (Smith et al., 1985).

2.8 Statistical analysis

Data analysis was conducted using the software GraphPad Prism (v9.5.1). Group comparisons were performed using Two-Way ANOVA followed by Tukey's test for all data except for the search strategy and number of errors acquired from the Barnes maze, which were analyzed using the Kruskal-Wallis test followed by Dunn's test. To examine the relationship between depressive-like behavior and biochemical markers, we performed Pearson correlation analyses between FST immobility and MDA, TNF-α, and BDNF levels. Because the distribution of BACE1 did not meet parametric assumptions, its association with FST was assessed using Spearman’s rank correlation. All p-values obtained from these correlation analyses were corrected for multiple testing using the original Benjamini–Hochberg

false discovery rate (FDR) method. Normality was assessed using Q-Q plots. Results were expressed as mean ± SEM, with $p < 0.05$ considered statistically significant.

3 Results

3.1 Effect of iTBS on spatial memory in MS rats

The analysis using two-way ANOVA indicated significant variations among groups in terms of the latency to find the specific target hole (Fig. 2A), which the rats had previously learned to locate ($F(1, 28) = 44.36$, $p < 0.001$) with significant interaction differences ($F(1, 28) = 50.75$, $p < 0.001$). The analysis revealed that MS significantly changed the time taken to locate the escape hole compared to the sham group ($p < 0.001$), while iTBS significantly reduced latency compared to the MS group ($p < 0.001$).

Additionally, the analysis indicated significant differences in errors made until finding the target hole (Kruskal-Wallis test, $H = 19.05$, $df = 3$, $p < 0.001$, Fig. 2B). The MS group made significantly more errors than the sham group ($p < 0.001$). However, iTBS treatment significantly decreased the number of errors relative to the MS group ($p < 0.05$).

Strategy scores (Fig. 2C), based on maze performance, were significantly shifted from spatial to random patterns in the MS group (Kruskal-Wallis test, $H = 11.91$, $df = 3$, $p = 0.008$). Magnetic stimulation significantly shifted the strategy scores toward spatial patterns compared to MS rats ($p < 0.05$).

3.2 Effect of iTBS on open field test in MS rats

In the open field test, there were significant differences observed across the groups ($F(1, 28) = 18.32$, $p < 0.001$, Fig. 3A) with significant interaction differences ($F(1, 28) = 4.795$, $p < 0.05$). Further analysis showed that MS significantly reduced the time spent in the center compared to the sham group ($p < 0.001$), while iTBS significantly increased center time compared to the MS group ($p < 0.05$).

3.3 Effect of iTBS on elevated plus maze test in MS rats

Spending less time in the open arms indicates higher anxiety levels (Fig. 3B). Analysis showed significant changes in EPM between groups ($F(1, 28) = 39.58$, $p < 0.001$) with significant interaction differences ($F(1, 28) = 9.823$, $p < 0.01$). Further analysis indicated that the MS rats spent significantly

less time in the open arms compared to the sham group ($p < 0.001$). iTBS significantly increased open-arm time relative to MS rats ($p < 0.01$).

3.4 Effect of iTBS on forced swim test in MS rats

FST was used to measure depression levels in our study (Fig. 3C). Two-way ANOVA analysis showed significant differences among groups in immobility time during the FST ($F (1, 28) = 15.30$, $p < 0.001$) with significant interaction differences ($F (1, 28) = 7.453$, $p < 0.05$). Immobility time during the FST was significantly increased in MS rats ($p < 0.001$), while iTBS significantly decreased immobility time compared to the MS group ($p < 0.01$).

3.5 Effect of iTBS on biochemical parameters in MS rats

Biochemical factors were analyzed for differences between groups using two-way ANOVA. Significant differences were found in oxidative stress markers: ROS ($F (1, 16) = 5.498$, $p < 0.05$), MDA ($F (1, 16) = 6.943$, $p < 0.05$), GSH ($F (1, 16) = 52.88$, $p < 0.001$), CAT ($F (1, 16) = 65.59$, $p < 0.001$), and SOD ($F (1, 16) = 17.88$, $p < 0.001$) with significant interaction differences ROS ($F (1, 16) = 4.920$, $p < 0.05$), MDA ($F (1, 16) = 9.783$, $p < 0.01$), GSH ($F (1, 16) = 24.47$, $p < 0.001$), CAT($F (1, 16) = 70.32$, $p < 0.001$) and SOD ($F (1, 16) = 10.21$, $p < 0.01$) (Fig. 4A - 4E). Further analysis showed that ROS and MDA levels were significantly increased in the MS group ($p < 0.05$ and $p < 0.01$, respectively), while GSH, CAT, and SOD levels were significantly reduced ($p < 0.001$). iTBS treatment partially reversed these changes, reducing ROS and MDA levels ($p < 0.05$ and $p < 0.01$, respectively) and significantly increasing GSH, CAT, and SOD levels ($p < 0.001$).

In terms of inflammation (Fig. 5A), the level of TNFα in the hippocampus showed significant changes between groups ($F (1, 16) = 10.88$, $p < 0.01$) with significant interaction differences ($F (1, 16) = 5.654$, $p < 0.05$). TNFα levels were significantly increased in the MS group ($p < 0.01$), and iTBS treatment significantly reduced TNFα levels to values not significantly different from the sham group ($p < 0.05$). The level of BDNF (Fig. 5B) also showed significant changes between groups ($F (1, 16) = 6.122$, $p < 0.05$) with interaction differences ($F (1, 16) = 4.563$, $p = 0.05$). In the MS group, hippocampal BDNF

levels were significantly reduced ($p < 0.05$), while treatment with iTBS significantly elevated hippocampal BDNF levels relative to the MS group ($p < 0.05$).

Analysis of BACE1 ($F (1, 16) = 13.52$, $p < 0.01$) activity (Fig. 5C) revealed a significant increase in the MS group compared to the Sham group ($p < 0.01$). However, our treatment reduced BACE1 activity toward Sham group levels ($p < 0.01$). Significant interaction differences ($F (1, 16) = 6.480$, $p < 0.05$)

3.6 Correlation analysis for FST and biochemical parameters

To assess the relationship between depressive-like behavior (FST immobility) and key biochemical parameters, we performed Pearson correlation analyses for MDA (key oxidative stress marker), TNFα, and BDNF. These analyses revealed significant correlations between FST and MDA ($r = 0.43$, $p = 0.029$), TNFα ($r = 0.48$, $p = 0.017$), and BDNF ($r = –0.39$, $p = 0.045$). Correlation between BACE1 and FST was evaluated using Spearman's rank correlation, which indicated a positive correlation ($r = 0.72$, $p < 0.001$).

4 Discussion

Our research showed that 10 days of iTBS had a therapeutic effect on early-life stress induced by MS to some extent. In this study, we showed that MS led to memory impairment and increased anxiety and depression later in this model. These behavioral symptoms were accompanied by oxidative stress, inflammation, and reduced BDNF levels in the hippocampus of MS rats. iTBS attenuated these MS-induced symptoms.

Early life stress can affect the hypothalamic-pituitary-adrenal (HPA) axis and subsequently influence behavior and brain structures such as the hippocampus (Huang, 2014). Research suggests the hippocampus is vulnerable to oxidative stress (Salim, 2017). MS can cause long-lasting damage to the hippocampus through mechanisms such as inflammation, eventually leading to serious mental problems (Aisa et al., 2007; Diehl et al., 2012; R. Wang et al., 2020). Consistent with other studies, our experiment showed that MS caused several behavioral problems, including increased latency to

find the target hole in the Barnes maze, reduced time in open arms in the EPM and increased immobility time in the FST.

As mentioned, MS can affect oxidative stress and inflammation, as shown in our study. An increase in ROS production activates cellular antioxidant defense system, and when this system fails, oxidative stress begins to increase (de Almeida et al., 2022; Sharma et al., 2023). Our study showed that ROS and MDA levels increased and the antioxidant system was disrupted. Another study reported a decrease in total brain antioxidants and an increase in MDA and nitrite levels resulting from MS (Rostami-Faradonbeh et al., 2024). Our study showed, that CAT, SOD, and GSH levels also decreased later in life after MS. BACE1 activity can be affected by oxidative stress elevation (Mouton-Liger et al., 2012). Our results showed increased BACE1 activity and cognitive impairments. Another study also showed MS involvement in hippocampus inflammation (Farzan et al., 2023). TNFα levels increased in our experiment as a marker of hippocampal inflammation in MS rats.

BDNF is an important factor that plays a role in neural connectivity and shaping neurons (Deinhardt & Chao, 2014). BDNF is also important in depression (Yu & Chen, 2011). In our study, the MS led to reduced levels of BDNF, consistent with previous reports (Ohta et al., 2017).

TBS is increasingly gaining attention because it requires less stimulation time (Stoby et al., 2022). There is evidence that iTBS has an antidepressant effect on rats, mediated by an increase in dendritic spine density and BDNF modulation (Lee et al., 2021). Previous studies have also reported that iTBS can enhance cognitive function in a rat model of Alzheimer's disease (J. Stanojevic et al., 2022). Our study showed that iTBS treatment after MS reduced depression-like behavior and partially improved memory.

Several studies report that rTMS has antioxidant effects. It has been reported that TMS stimulation enhanced the effectiveness of the antioxidant system of cells and reduced oxidative stress (Medina-Fernández et al., 2018). It was also reported that magnetic stimulation has anti-inflammatory properties

(Tian et al., 2020). Both effects can reduce MS-induced early-life stress damage, as observed in our study.

iTBS also has the ability to modulate BDNF levels, as observed in our study. Similarly, another study reported that long-term iTBS treatment in an ischemic reperfusion model can improve recovery through the miR-551b-5p/BDNF/TrkB pathway (L. Wang et al., 2022). This aligns with other related studies showing that rTMS increases BDNF levels and enhances cognition (Shang et al., 2016).

This study has several limitations; the most important is the coil size, which was relatively large for rat brains at this age and may have influenced other brain structures and networks, potentially affecting results. Another limitation is this study lacks any immunohistochemical or histological analyses; future studies could benefit from these types of analyses to demonstrate the effects of this technique on brain structures. Another limitation is that training data from the Barnes maze were not included; as only probe test data were used, conclusions about spatial learning are not fully supported. Males and females respond differently to acute and chronic stress regarding hormone release and cognitive functions. In this respect, females often display greater resilience to chronic stress (Luine et al., 2017). Also, sex differences were not the main objective of our study; future studies are recommended to consider this aspect as well. Another limitation is that we tested the therapeutic effect of iTBS with certain tests that may have different outcomes and this important issue should be considered for future studies.

Taken together, our data suggest that iTBS has therapeutic effects on depressive-like behavior induced by MS in male rats. Ten days of iTBS improved spatial memory, reduced oxidative stress and inflammation, and increased BDNF levels in the hippocampus.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, Author names [MR, MA]; Methodology, Author names [GPJ, MA, MR]; Investigation, Author names [GPJ, MA, MR, AG]; Writing – Original Draft, Author names [GPJ];

Writing – Review & Editing, Author names [all authors]; Resources, Author names [MR, MA, SG]; Supervision, Author names [MR, GPJ]

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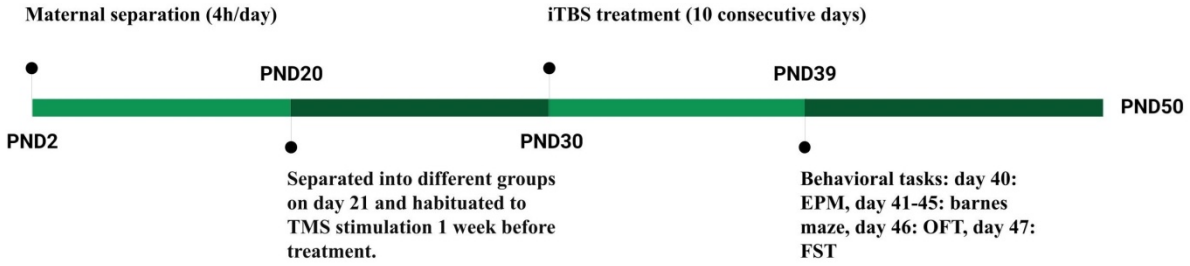
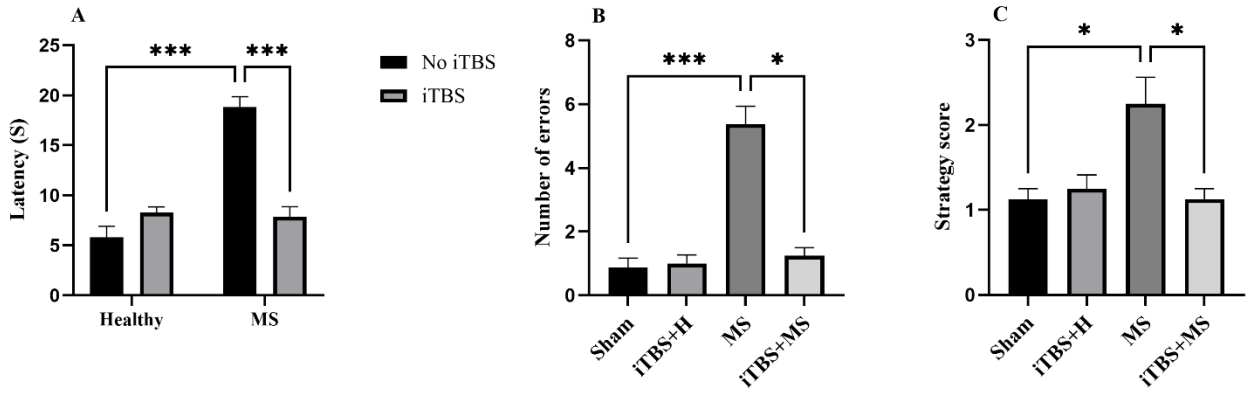


Fig.1



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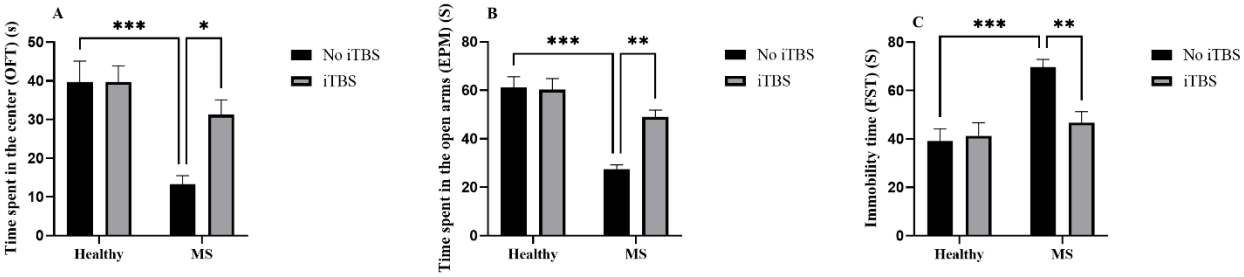

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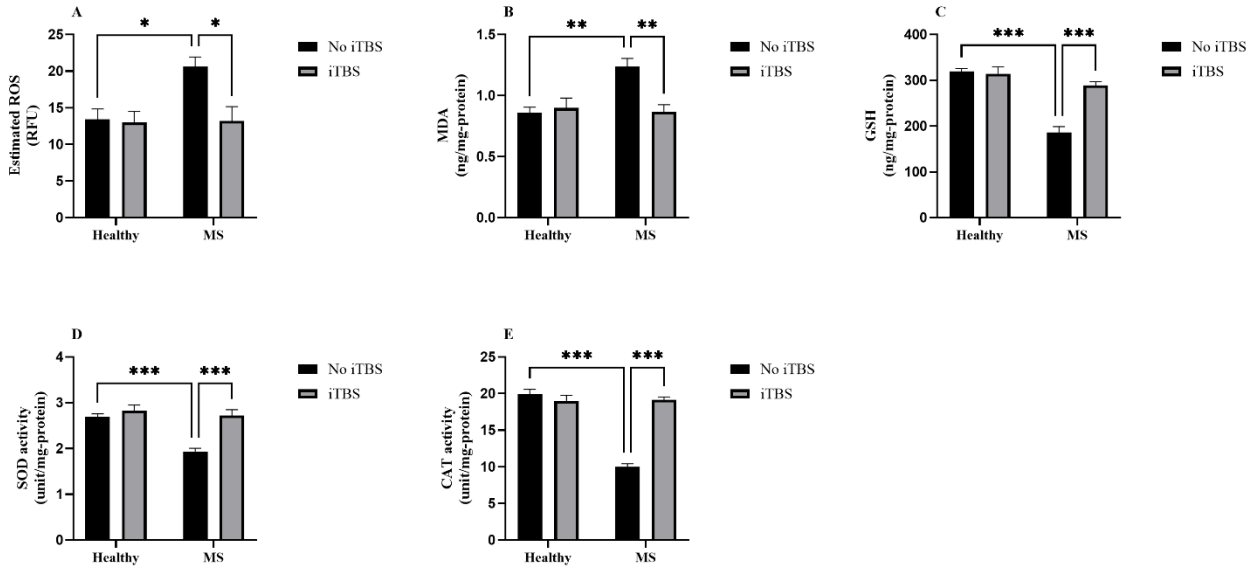

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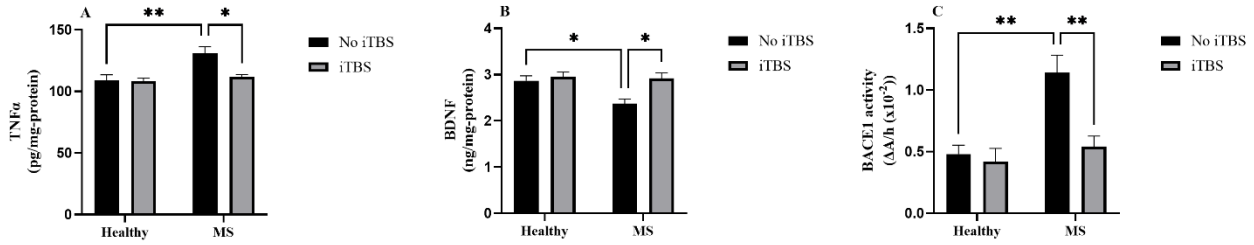

Fig.5

Figures legends

Figure 1: Timeline of This Study

Postnatal Day 2 (PND2) to PND20: Rats undergo maternal separation to induce depressive-like disorder.

Day 21: Male rats are randomly divided into groups.

PND30: iTBS treatment starts and continues for 10 consecutive days.

PND40: Behavioral evaluations begin.

PND50: Rats are sacrificed, and brain tissue is collected for further analysis.

Figure 2: The Effect of Intermittent Theta Burst Stimulation Treatment on Spatial Memory with Barnes Maze in Rat Model of Depression-Like Behavior Induced by Maternal Separation

A) Time spent to find the target hole in the probe test

B) Number of errors made to find the target hole in the probe test

C) Strategy score based on how the rats managed to find the target hole in the probe test

All data are presented as mean ± SEM (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001

Figure 3: The Effect of Intermittent Theta Burst Stimulation Treatment on open field test (OFT), Elevated Plus Maze (EPM) and Forced Swim Test (FST) in Rat Model of Depression-Like Behavior Induced by Maternal Separation

A) Time spend in the center showing the anxiety level in the open field test

B) Time spent in the open arms of the EPM

C) Immobility time in water during the FST

All data are presented as mean ± SEM (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001

Figure 4: The Effect of Intermittent Theta Burst Stimulation Treatment on Oxidative Stress in Rat Model of Depression-Like Behavior Induced by Maternal Separation

A) Reactive oxygen species

B) Malondialdehyde

C) Glutathione

D) Superoxide dismutase

E) Catalase

All data are presented as mean ± SEM (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001

Figure 5: The Effect of Intermittent Theta Burst Stimulation Treatment on Other Biochemical Parameters in Rat Model of Depression-Like Behavior Induced by Maternal Separation

A) Tumor necrosis factor alpha

B) Brain-derived neurotrophic factor

C) Beta-secretase 1 (BACE1)

All data are presented as mean ± SEM (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001