Effects of minocycline on neuroinflammation, gut microbiome and hippocampal neurogenesis in rats with Gulf War Illness

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Research

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Abstract

Background

Accumulating evidence suggests that deficits in neurogenesis, chronic inflammation and gut microbiome dysregulation contribute to the pathophysiology of Gulf War Illness (GWI). Minocycline has been demonstrated to be a potent neuroprotective agent and could regulate neuroinflammation. The present study intended to investigate whether treatment of minocycline maintain better cognition and mood function in a rat model of GWI and the potential mechanism.

Methods

Rats received 28 days of GWI-related chemical exposure and restraint stress, along with daily minocycline or vehicle treatment. Cognitive and mood function, neuroinflammation, neurogenesis and gut microbiota were detected.

Results

We found that minocycline treatment induced better cognitive and mood function in a GWI rat model, as indicated by open-field test, elevated plus maze test, novel object recognition test and forced swim test. Moreover, minocycline treatment reversed the altered gut microbiome, neuroinflammation and the decreased hippocampal neurogenesis of rats with GWI.

Conclusion

Taken together, our study indicated that minocycline treatment exerts better cognitive and mood function in GWI rat model, which is possible related to gut microbiota remodeling, restrained inflammation and enhanced hippocampal neurogenesis. These results may establish minocycline a potential prophylactic or therapeutic agent for the treatment of GWI.

1. Introduction

Gulf War Illness (GWI) is a multi-symptom illness that affects 30% of veterans from the 1991 Gulf War and is characterized by a spectrum of chronic medically unexplained symptoms including fatigue, reduced ability to concentrate, insomnia, dizziness, functional gastrointestinal disorders, diminished cognitive and mood function [1]. The etiology and pathogenesis of GWI is very complex and may be related to veterans' experience in wartime, vaccination, psychological stress, infectious diseases, and exposure to various chemicals, including lean uranium, oil well flame smoke, nerve gas, pyridostigmine bromide (PB) and insecticides [2]. Currently, there is increasing evidence suggesting that PB and pesticides

are closely associated with the onset of GWI [3]. These studies also suggested that a majority of veterans with GWI used higher amounts of PB pills and pesticides during the Gulf War. However, to data, there is no therapies available for GWI.

Consistent with the above findings, studies on animal model of GWI also have found that insecticides such as N, N-diethyl-m-toluamide (DEET) and permethrin can cause chronic brain dysfunction, including decreasing adult hippocampal neurogenesis, over activation of neuro-inflammation, damaging the blood-brain barrier, causing nerve cells death in the dentate gyrus and hypothalamus [4]. Brain dysfunction is typified by depression, anxiety and impaired cognition [5]. Meanwhile, it is well-known that adult hippocampal neurogenesis plays a crucial role both in cognitive function and mood regulation [6]. Importantly, the impairment of adult hippocampal neurogenesis contributes to several psychiatric disorders, such as GWI, addiction and depression[5,7]. Brain imaging studies of GWI patients suggested that the scores on immediate and delayed verbal and visual retrieval decreased in GWI patients, and the hippocampal volume, especially the hippocampal head, was significantly smaller than that of the healthy civilian [8]. Additionally, over activation of neuroinflammation have been identified in animal models of GWI using exposures to several chemical combinations [9]. For GWI patients, the levels of multiple proinflammatory biomarkers in the blood also increased significantly [10]. More importantly, enhanced neurogenesis, and alleviation of neuroinflammation by curcumin treatment could leads to better cognitive and mood function in GWI rats [11]. Taken together, the results of these studies have indicated that over activation of neuroinflammation and impaired neurogenesis in the adult hippocampus might be the key biological processes involved in GWI pathophysiology and may as the potential therapeutic targets for treating GWI.

Minocycline, as a long-acting tetracycline agent, has a profound neuroprotective effect associated with the improvement of cognitive function, mood regulation and neurogenesis during neuroinflammatory conditions. Several studies have reported that minocycline was beneficial in neurological models such as schizophrenia and depression [12,13]. However, the role of minocycline in promoting hippocampal neurogenesis and alleviating of neuroinflammation, including potential cognitive and mood enhancing properties in GWI, remains unclear. We here aimed to evaluate the effects of minocycline treatment on neuroinflammation and neurogenesis in the hippocampus in the rat model of GWI.

2. Materials And Methods

2.1 Animals and groups

Adult male Sprague-Dawley rats (9 weeks old) were used. The experiments were approved by the Animal Care and Use Committee of the General Hospital of Northern Theater Command and was in consistence with the principles outlined in the National Institutes of Health Guide. Rats were randomly divided into the following groups: Control group, GWI group, and GWI + Mino group.

2.2 Exposure of rats to PB, Permethrin and restrained-stress

The pyridostigmine bromide (PB) was purchased from Sigma (Burlington, MA, USA) and was administered by oral gavage at a dose of 1.3 mg/kg for 28 d (in 500 μL water). The rats exposed to chemicals DEET (in 200 μL 70% alcohol, 40 mg/kg) and PM (in 200 μL 70% alcohol, 0.13 mg/kg) over shaved skin areas located on the back of the neck and the upper thoracic region for 28 consecutive days. The doses of PB, PM, and DEET were chosen according to previous reports.[5] Moreover, 5 min of daily restraint stress was conducted for 28 days by a rat restrainer, as previously reported.[14]

2.3 Administration of minocycline and BrdU labeling

As Fig. 1 show that rats received PB, Permethrin and restrained-stress exposure and with normal saline intraperitoneal or minocycline (Sigma) dissolved in sterile saline, at dose of 40 mg/kg/day (minocycline) by intraperitoneal administration for 28 days. Rats were received BrdU at 50 mg/kg body weight dose intraperitoneally, for a total of 3 injections in 3 days.

2.4 Behavioral experiments

Open field test: The open-field test was performed according to previous methods [15]. Briefly, rats were placed in the center of the open-field box for a period of 10 min and the activity of rats was recorded by a video camera. The time spent in the center and distance traversed in the TTcenter arena were analyzed using Ethovision 11.0 (Noldus).

The elevated plus maze test: The elevated plus maze test was performed according to a previous report [14], and the duration of time for the rat to enter any of the 4 arms was recorded when all 4 paws crossed from the central region into an arm during the 10 min testing period.

Novel object recognition test: The novel object recognition test paradigm consisted of mainly two phases, including the adaptive phase and test phase. The duration of the rat explores new object (B object) and old object (A object) were recorded and analyzed by Ethovision 11.0 (Noldus). As the distance between the tip of nose and object is less than 2 cm, definite as exploration time. Exploration preference index = time for mice to explore new objects (B) / total time for mice to explore all objects (A + B) * 100%.

Forced swim test: Forced swim test is widely used for measurement of depression-like behavior in rodents [16]. Briefly, rats were individually placed into a cilindrical vessels (height: 46 cm, diameter: 30 cm) for 6 min, which containing 30 cm height of water (25 °C). Total time spent immobile of rats (adopts an immobile posture) during the last 4 min of the 6 min test were recorded.

2.6 Immuno-fluorescence

Immuno-fluorescence was conducted according to previous method [15]. Briefly, serial coronal brain sections (5 or 40 μm in thickness) were collected. The sections were first washed in 0.01 M PBS to remove the cryoprotectant solution. The sections were then incubated with primary antibodies for 12 h at 4 °C, including rabbit anti-NeuN (1:1000, BD, San Jose, CA) or goat anti-DCX (1:200, Santa Cruz Biotechnology, CA, USA). For BrdU staining, all the sections were pretreated with 2 N HCl for 1 h at 37 °C

to denature the DNA followed by 0.1 M borax (pH 8.5) treatment for 10 min to neutralize before the regular immunostaining procedure. After washing, the sections were incubated with biotinylated secondary antibody (1:200, Dako, Glostrup, Denmark) (2 h, 37 °C), followed by the avidin-biotin complex (Dako). Finally, sections were analyzed under a Lycra microscope.

2.7 Quantitative RT-PCR

The TRIZOL reagent (Sigma Aldrich) was used to extract total RNA according to the manufacturer's instructions and reverse transcripted to obtain cDNA using a PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan). Realtime PCR was performed using cDNA samples with YBR Green qPCR Mix (Takara Bio Inc., Shiga, Japan) with a CFX96 Real-time PCR System (Bio-Rad). Cq values were normalized to using the housekeeping gene of GAPDH. Relative gene expression levels were calculated using the $2 - \Delta\Delta C(t)$ method [17].

2.8 ELISA

The concentration of rat IL-10 (Wuhan USCN) and IL-17 (Wuhan USCN) in the hippocampus and serum were measured by their respective ELISA kits according to the manufacturers' instructions. The results are expressed as picograms milligram (pg/mg) or picograms permilliliter (pg/ml).

2.9 Western blotting

The protein concentration of hippocampus was detected with a BCA kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The proteins were separated on 15% polyacrylamide gels by standard SDS polyacrylamide gel electrophoresis at 80 V for 120 min, then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The PVDF membranes were blocked with 5% non-fat dry milk in 0.05% Tween-20 in PBS for 1 h at room temperature. Membranes were incubated with primary antibodies against TLR4 (1:1000, Santa Cruz Biotechnology), NF-κB p65 (1:2000, BD Biosciences), β-actin (1:2000, Cell CWBIO) for 12 h at 4℃. The membranes were further incubated with horseradish peroxidase conjugated secondary antibody (1:1000, Santa Cruz Biotechnology) for 2 h at 37℃. Bound antibodies were visualized using a chemiluminescence detection system. The signals were measured by scanning densitometry and computer-assisted image analysis.

2.10 Microbiome analysis

Fecal pellets and luminal contents were collected from the animals of each group after sacrifice, and then bacterial DNA from cecal contents was extracted using a DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. 16 s rRNA gene sequencing of fecal DNA samples was performed by NovaSeq sequencing platform. The V3-V4 region was amplified and sequencing was done using an Illumina HiSeq sequencing platform. Sequences from all samples were processed using QIIME (Knight and Caporaso labs) and then assigned by the RDP classifier (Michigan State University) against the Greengenes database.

2.11 Statistical analyses

All data are presented as the mean ± SEM and analyzed using SPSS

20.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way ANOVA followed by Tukey's post hoc tests. For all comparisons, the significance level was set at $P < 0.05$.

3. Results

3.1 Minocycline treatment alleviated neurobehavioral deficits in a GWI rat model

A timeline with the experimental design was shown in Fig. 1A. As previous studies reported that animals with GWI exhibited increased levels of depressive- and anxiety-like behaviors [5]. We found that minocycline treatment reverses decreased time in the central area (Fig. 1B) for GWI rats and decreased distance in the central area (Fig. 1C) in the open field test. Meanwhile, the results from the elevated plus maze test showed that rats in GWI + Mino group displayed higher level in the percentage of time spent in open-arms (Fig. 1D) and percentage of open arm entries (Fig. 1E), compared with rats in GWI group. Moreover, we revealed that GWI rats showed increased immobility in the forced swim test, compared with control rats. While minocycline treatment reversed this effect significantly in GWI rats (Fig. 1F). These results indicated that minocycline treatment decreases depressive- and anxiety-like behaviors in GWI rats.

To test whether minocycline corrected recognition memory deficit in GWI rats, we performed the novel object recognition test. The results showed that GWI rats with minocycline treatment for 28 days significantly improved recognition memory compared to that of the vehicle-administered GWI group (Fig. 1G). Meanwhile, the total exploratory time of all objects (Fig. 1H) or the total distance moved in the box (Fig. 1I) were comparable across the three groups, indicating that the results are not influenced by potential changes in overall activity or lack of motivation. Thus, these findings implied that minocycline treated GWI rats exhibited better recognition memory.

3.2 Minocycline treatment enhanced hippocampal neurogenesis in GWI rats

The hippocampal pathology of GWI typified by decreased neurogenesis, while enhanced hippocampus neurogenesis could alleviate GWI-related behaviors [5,11]. Therefore, we analyzed the proliferating cell population in the SGZ-GCL using the BrdU incorporation assay, which demonstrated that a reduced density of BrdU + cells in GWI rats compared to control group. While minocycline treatment rescued the decreased BrdU + cells in the SGZ-GCL of GWI rats significantly (Fig. 2A-B). Furthermore, we analyzed the percentages of BrdU + cells expressing DCX in the SGZ-GCL at 14 days following BrdU injections. We found that no difference in the percentages of BrdU + cells expressing DCX between the three groups (Fig. 2C-D). While, the DCX + cells decreased significantly in GWI rats compared to control rats, which indicated that the number of newly born immature neurons were diminished in the GWI rats, while this effect was blocked by minocycline treatment (Fig. 2E). Besides, we further analyzed the percentages of

BrdU + cells expressing NeuN in the SGZ-GCL at 28 days following BrdU injections. The data showed that no difference in the neuronal fate-choice decision by newly born cells between the three groups (Fig. 2F-G).

3.3 Minocycline treatment attenuated chronic systemic and brain inflammation in GWI rats

Previous studies revealed that exposure to Gulf war illness-related chemicals resulted in the immune system dysfunction [10]. Our results showed that the concentration of IL-17 was elevated both in serum and hippocampus of GWI rats and this increase was attenuated by minocycline treatment (Fig. 3A, B). Moreover, the level of the anti-inflammatory marker of IL-10 in serum and hippocampus of GWI rats was significantly higher than the rats in the control group, but minocycline treatment increased the level of IL-10 significantly for GWI rats (Fig. 3C, D).

Furthermore, as previous studies reported that the TLR4/NF-κB signaling pathway is involved in the process of neuroinflammation [18]. Thus, to reveal the mechanism of minocycline treatment for regulation of neuroinflammation, we detected the expression of TLR4 and NF-κB p65 signaling pathway in hippocampus of rats after minocycline treatments by quantitative RT-PCR and western blotting (WB). Our results showed that the expression of TLR4 and NF-κB p65 were elevated significantly in GWI rats and these increases were attenuated by minocycline treatment (Fig. 3E-H).

3.4 The effects of minocycline treatment on microbial diversity and the microbial community in GWI rats

We analyzed the composition of rat gut microbiota in stool samples by next-generation sequencing using V3–V4 hyper-variable 16S rRNA genomic region. Compared with the Control group, the GWI group had significantly increased commensal richness and diversity, as confirmed by the increased ACE index (Fig. 4A) and Shannon diversity (Fig. 4B). The ACE index and Shannon diversity was decreased in the GWI + Mino group compared with the GWI group (Fig. 4A-B). We also evaluated the dissimilarity between cecum bacterial communities among the groups by using principal coordinate analysis (PCoA) of the weighted UniFrac distance. The results showed that minocycline treatment reshaped the microbiota of rats with GWI (Fig. 4C). Moreover, we found that there were significant differences in the composition of gut bacteria both at the class and family levels between the groups (Fig. 4D-E). The class *Bacilli, Erysipelotrichia* and *Deltaproteobacteria* were underrepresented and *Bacteroidia, Gammaproteobacteria, Spirochaetia* and *Mollicutes* were overrepresented in GWI group compared with Control group, while minocycline treatment rescued these effects for rats with GWI (Fig. 4D). In addition, the abundance of the partial families that increased in GWI group, including *Ruminococcaceae, Succinivibrionaceae, Lachnospiraceae, Prevotellaceae* and *Muribaculaceae, but Lactobacillaceae, Peptostreptococcaceae* and *Erysipelotrichaceae* decreased. These changes in GWI rats also were rescued by minocycline treatment (Fig. 4E).

4. Discussion

In this study, we provided new evidence that minocycline treatment alleviated impaired cognitive and mood function in a rat model of GWI. Specifically, we observed that these functional benefits may are paralleled by changes in neuroinflammation, hippocampal neurogenesis and gut microbiome. To the best of our knowledge, our report is the first to present comprehensive evidence that minocycline treatment is efficacious to alleviate GWI-related behaviors.

It has been shown that impairments in mood and cognitive function are the key brain abnormalities observed both in veterans with GWI and animal models [5]. The hippocampal pathology of GWI mainly include decreased neurogenesis, partial loss of principal neurons, mild inflammation and reduced hippocampal volume [5]. These changes in hippocampus showed an association with mood and cognitive dysfunction in a model of GWI. There are credible evidences to support the hypothesis that adult hippocampal neurogenesis in the hippocampus contributes to mood regulation. Especially, in some rodent models of depression and patients, reduced the size of hippocampus may be related to decreased neurogenesis and mature neuronal cell loss [19]. Moreover, previous research has indicated that enhanced neurogenesis leads to better cognitive and mood function in a model of GWI [5]. Consistented with these findings, we found minocycline treatment alleviated depressive- and anxiety-like behaviors and recognition memory in GWI rats. Meanwhile, substantial decreases in hippocampal neurogenesis were found in a model of GWI, while enhance hippocampal neurogenesis has been observed after minocycline treatment in rats with GWI. Thus, it is likely that the effects of minocycline on GWI-related behaviors may involve in enhance hippocampal neurogenesis.

Accumulating evidences suggest that chronic inflammation play a crucial role in regulating hippocampal neurogenesis [20]. Especially, neuroinflammation is one of the most essential factors in the progression of GWI. As previous reports, excessive neuroinflammation presented in veterans suffering from GWI and also observed in animal model of GWI [10]. Part of serum proteins associated with inflammation were significantly increased in veterans suffering from GWI, including interleukin 6, C-reactive protein, matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) [21]. In animal model of GWI, suppression of the inflammatory responses showed a neuroprotective effect, improved neurogenesis, and better cognitive and mood function [22]. Previous research has indicated that excessive neuroinflammation showed negative effect on neurogenesis in the hippocampus [23]. Interestingly, in the present study, we found minocycline treatment suppressed neuroinflammation in association with enhanced neurogenesis, better cognitive and mood function in animal models of GWI, indicating a linkage between these processes. Moreover, TLR4/NF-κB pathway in the most important regulators of inflammatory cascades in neuroinflammation. In our study, we have observed that minocycline treatment can reverse increased levels of TLR4 and NF-κB in hippocampus of rats with GWI. Consistently, previous studies also have revealed the anti-inflammatory effect of minocycline in other models of neurodegenerative and neuroinflammatory diseases, including Alzheimer's disease, depression, intracerebral hemorrhage and

etc.[24] Thus, our results suggest that minocycline show an anti-inflammatory effect by interrupting of the TLR4/NF-κB signaling pathway.

Studies have shown that stress can change the composition of gut microbiota, and disruptions of the gut microbiota contribute to the changes of systemic inflammation, behaviors, and cognitive function [25]. In detail, previous studies have revealed that disruptions of the gut microbiota can decrease the number of regulatory T cells (Treg cells), and leading to an increase in interleukin (IL)-17-positive γδ T cells through altered dendritic cell activity in small intestine [26]. Moreover, IL-10 and IL-17 are required for the neuroprotection afforded by intestinal dysbiosis. Further, increasing the number of Treg cells in the intestine can reduce the migration of intestinal of effector T cells from the gut to the leptomeninges, thereby reducing the inflammation of brain. Firas et al. have revealed that several gram negative bacterial genera were increased in an established rodent model of GWI [27]. Gut dysbiosis with simultaneous leaky gut and systemic endotoxemia-induced TLR4 activation contributes to GWI chemical-induced neuroinflammation and gastrointestinal disturbances. Evidence found in our study showed that a direct relationship of gulf war chemical exposure and altered gut microbiota diversity with changes in the Phylum and family levels.

In summary, our study provided the first evidence that minocycline treatment alleviated impaired cognitive and mood function via reversing gut dysbiosis, the dysregulation of the neuroinflammation and restoring hippocampal neurogenesis in a rat model of GWI. Our study suggested that close attention should be paid to the link between GWI and dysbiosis. Our findings provide evidence that minocycline can be a novel strategy that are efficacious for repairing gut dysbiosis, suppressing inflammation and enhancing neurogenesis may be helpful for improving mood and cognitive function in veterans with GWI.

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Liang Liu and Yan Lv performed animal study and wrote the main body of the manuscript. Hui-Sheng Chen provided critical writing in the revised manuscript. Cheng Du and Yan Lv designed and instructed the writing of the manuscript. All authors read and approved the final manuscript.

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Figures

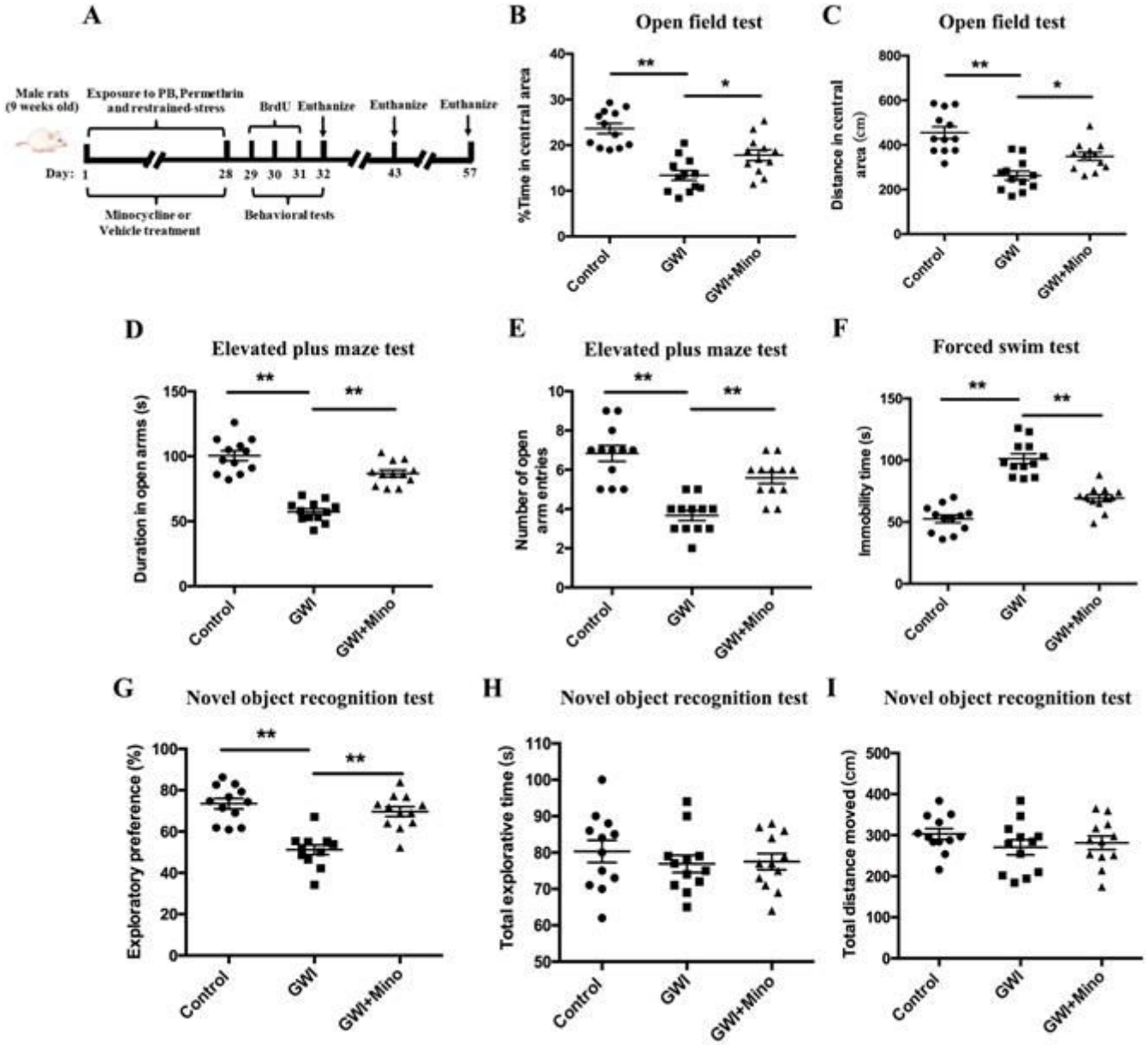

Figure 1

Minocycline treatment alleviated novel object recognition memory and mood function deficits in Gulf War Illness (GWI) rats. (A) Schematic of the experimental design. (B, C) Time spent in the center and distance traversed in the center arena were measured in the open field test among the three groups (n = 12). (D, E) Behavior of the three groups of rats in the elevated plus-maze test (n = 12). (F) Rats in GWI group displayed more immobility time on the forced swim test, and treatment with minocycline reversed this effect (n = 12). (G) Rats in GWI group displayed a lower exploratory preference compared to control rats, and the treatment of minocycline reversed this effect (n = 12). (H, I) There were no significant differences between three groups in the total explorative time and the total moved distance (n = 12). **P < 0.01.

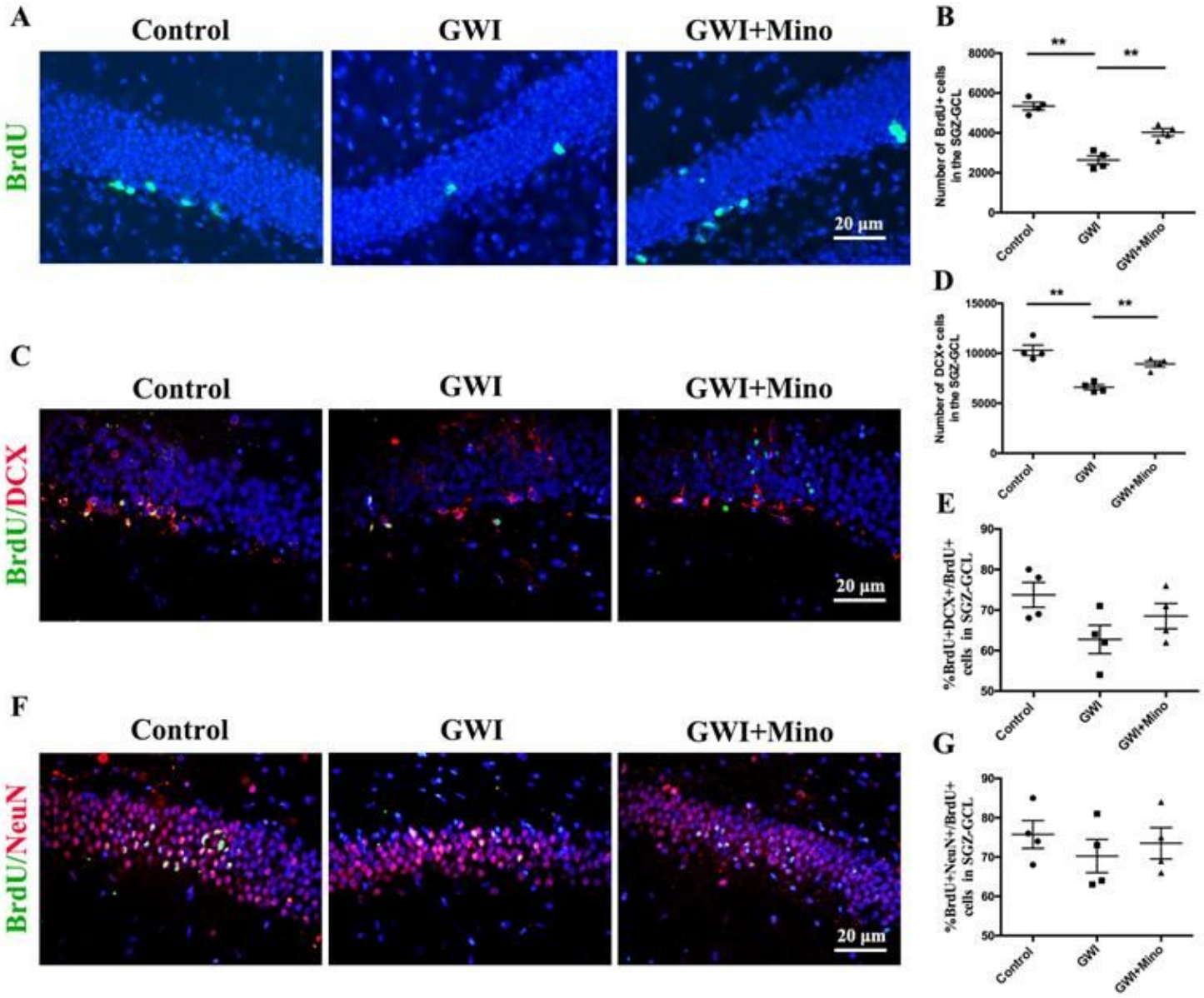

Figure 2

Minocycline treatment enhanced hippocampal neurogenesis in rats with Gulf War Illness (GWI). (A, B) BrdU-positive cells in the subgranular zone-granule cell layer (SGZ-GCL) for each of the three groups (n=4). (C-E) BrdU-positive cells and DCX-positive cells in the SGZ-GCL (n=4). (F-G) There were no significant differences between three groups in the percentages of BrdU-positive newly born cells that differentiate into NeuN-positive neurons (n=4). **P < 0.01.

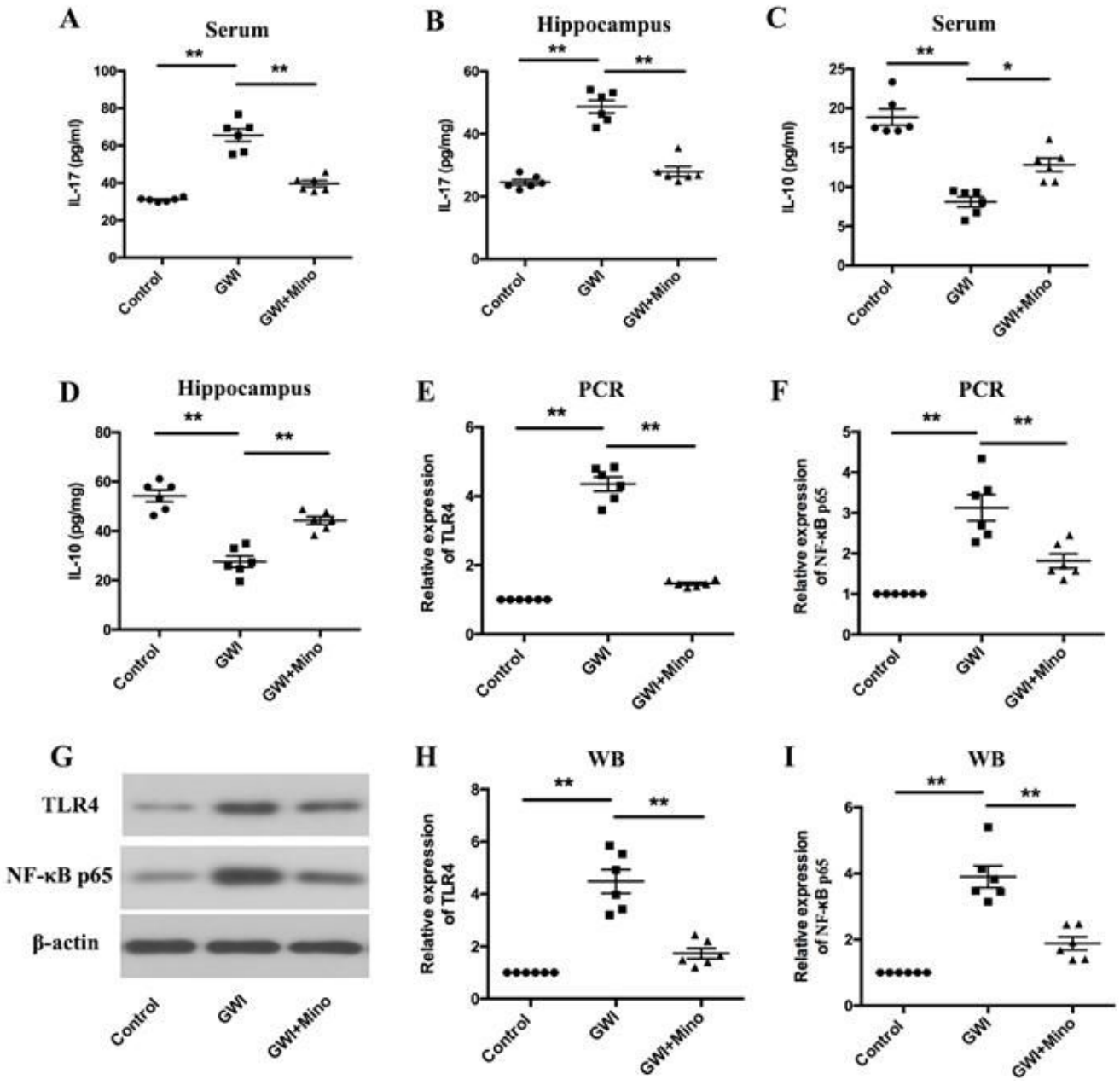

Figure 3

Minocycline treatment attenuated chronic systemic and brain inflammation in rats with Gulf War Illness (GWI). (A-D) The pro-inflammatory marker of IL-17 and anti-inflammatory markers of IL-10 in serum and hippocampus (n=6). (E-I) The expression of TLR4 and NF-κB p65 in hippocampus of different groups rats were detected by PCR and western blotting (n=6). *P < 0.05, **P < 0.01.

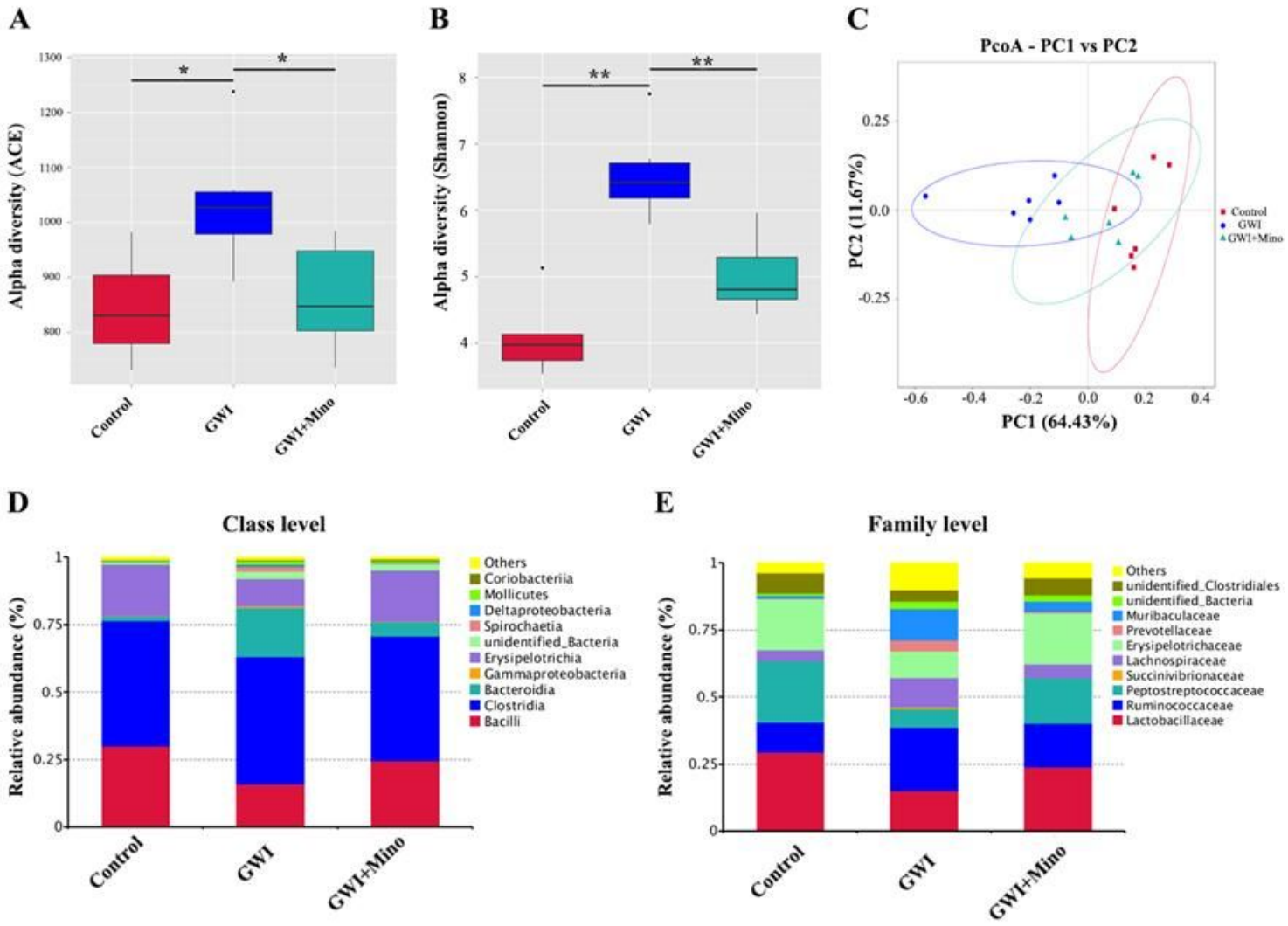

Figure 4

Minocycline treatment rescued gut dysbiosis in rats with Gulf War Illness (GWI). (A, B) The microbial alpha diversity differences between three groups (ACE and Shannon diversity). There were observed significant increase in ACE and Shannon diversity of GWI over Control group, while minocycline treatment rescued these effects significantly (n=6). (C) The PCoA plot showing the microbial composition among three groups based on the weighted UniFrac distances (n=6). (D, E) The relative abundance of specific bacteria at the class level and family level for rats in different groups (n=6). *P < 0.05, **P < 0.01.