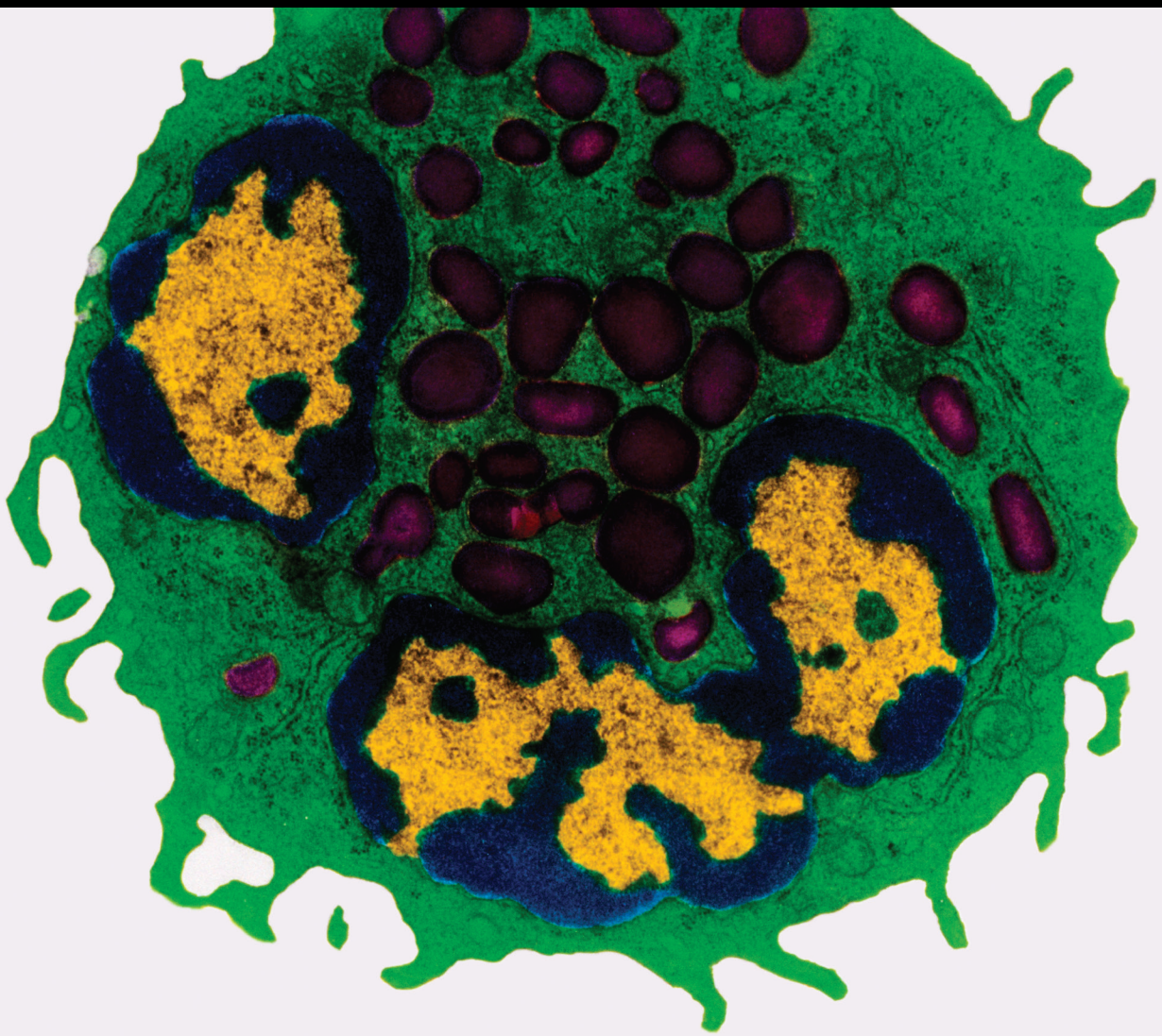
Mediators of Inflammation

Connection between Inflammation and Psychiatric Disorders: Exploring the Mechanism and the Potential Treatment Targets

Lead Guest Editor: Junhui Wang
Guest Editors: Hongxing Wang, Sun-Young Hwang, and Gang Luo



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Corrigendum

Corrigendum to "miR-221 Alleviates the Ox-LDL-Induced Macrophage Inflammatory Response via the Inhibition of DNMT3b-Mediated NCoR Promoter Methylation"

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In the article titled "miR-221 Alleviates the Ox-LDL-Induced Macrophage Inflammatory Response via the Inhibition of DNMT3b-Mediated NCoR Promoter Methylation"[1], the authors Wenjun Zeng, Yanan Duan, and Zhihua Yang were incorrectly affiliated to the institution "Department of Cardiology, Tongren Hospital, Yunnan 650032, China." The correct affiliation for these authors is "Department of Cardiology, 920th Hospital of PLA Joint Logistic Support Force, Yunnan 650032, China."

The corrected affiliations are shown in the author information above.

References

[1] J. Ye, Y. Wu, R. Guo et al., "miR-221 alleviates the Ox-LDL-induced macrophage inflammatory response via the inhibition of DNMT3b-mediated NCoR promoter methylation," *Mediators of Inflammation*, vol. 2019, Article ID 4530534, 15 pages, 2019.

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Research Article

Alterations in the Levels of Growth Factors in Adolescents with Major Depressive Disorder: A Longitudinal Study during the Treatment with Fluoxetine

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Guest Editor: Hongxing Wang

Major depressive disorder (MDD) has a prevalence of 5% in adolescents. Several studies have described the association between the inflammatory response and MDD, but little is known about the relationship between MDD and growth factors, such as IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF. It must be appointed that there are scarce reports on growth factors in adolescents with MDD and even fewer with a clinical follow-up. In this work, we evaluated the levels of growth factors (IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF) in MDD adolescents and the clinical follow-up during eight weeks of treatment with fluoxetine. *Methods*. All patients were diagnosed according to the DSM-IV-TR, and the severity of the symptoms was evaluated using the Hamilton Depression Rating Scale (HDRS). Growth factors IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF were quantified by cytometric bead array using serum samples from 22 adolescents with MDD and 18 healthy volunteers. *Results*. All patients showed clinical improvement since the fourth week of pharmacological treatment according to the HDRS. Considerably higher levels of IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF were detected in MDD adolescents as compared to healthy volunteers. A significant but temporal decrease was detected in basic FGF, G-CSF, and GM-CSF at week four of fluoxetine administration. *Conclusions*. To the best of our knowledge, this is the first report to show alterations in the levels of growth factors, such as IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF in MDD adolescents during eight weeks of clinical follow-up. These disturbances might be involved in the physiopathology of MDD since such growth factors have been proven to participate in the neural development and correct functioning of the CNS; therefore, subtle alterations in it may contribute to MDD.

1. Introduction

Major depressive disorder (MDD) is one of the mood alterations with the highest occurrence in adolescence [1]. Its onset during this stage of life is an essential risk factor for suicide and represents the second most common cause of death among teenagers [2]. It has been estimated that 2.8 million adolescents between the ages of 12 and 17 have experienced a depressive episode. MDD causes social, familial, and educational deterioration, which leads to a high rate of disability among this population [3]. Although several works on MDD describe alterations in the levels of cytokines [4], chemokines [5], growth factors [6], neurotransmitters, and hormones, there are few reports with a clinical follow-up in adolescents [7, 8]. In a previous work, we reported inflammatory profiles that include IL-2, IFN-γ, IL-1β, TNF-α, IL-6, IL-12, and IL-15, as well as IL-4, IL-5, IL-13, IL-1Ra, and IL-10 cytokines in depressed adolescents [8].

Soluble inflammatory mediators are molecules that not only activate and promote an efficient immune response [9] but also regulate neural maintenance and development throughout life [10]. A particular group of proinflammatory cytokines is involved in the upkeep of this balance since they can act as both hematopoietic and neurotrophic growth factors [6]. Growth factors control embryonic and postnatal states, promoting the adequate functioning of the central nervous system (CNS),therefore, subtle changes in the secretion and expression patterns of these factors are relevant to the physiopathology of MDD. There are descriptions of alterations in cognitive, emotional, and motor processes associated with a decrease in the levels of BDNF, EGF/ErbB3, EPO, IGF, NGF, TGF-β, and VEGF in peripheral blood [6]. The pharmacological treatment with monoamine oxidase (MAO) inhibitors, selective serotonin reuptake inhibitors (SSRIs), tricyclic agents, and serotonin and noradrenaline reuptake inhibitors (SNRIs) restores the levels of BDNF, NGF, FGF, TGF-β, VEGF, and IGF-1, as well as the expression of EGF [11–16]. Together, these findings have led to the consideration of growth factors as possible biomarkers for the diagnosis and prognosis of MDD [6]. In this work, we show significant alterations in the serum levels of seven growth factors in adolescent MDD patients during 8 weeks of pharmacological treatment with an SSRI (fluoxetine).

2. Methods

2.1. Patients. The study was performed in the National Institute of Psychiatry "Ramón de la Fuente Muñiz" in Mexico City, Mexico. We recruited 336 adolescents with MDD from January 2006 to December 2008, and 22 of them participated in the study based on the inclusion criteria. The inclusion criteria included men and women aged between 14 and 19 years who met the diagnostic criteria for major depressive disorder (MDD) per the DSM-IV-TR and had a minimum baseline score on the HRSD ≥ 14 and no history of treatment for MDD with SSRIs and whose current episodes were moderate, lasting no more than two years. All the patients who were included agreed to participate in the study (INPRF-2035) and signed informed consent forms. The patients were recruited per the clinical follow-up in the INPRF-2035 research protocol, as approved by the ethics committee of the National Institute of Psychiatry "Ramón de la Fuente Muñiz," Mexico.

2.2. Healthy Volunteers. Eighteen healthy volunteers (HVs) were recruited from the general population between January 2006 and December 2008. Clinical parameters of the HVs were within normal reference ranges (data not shown). The MINI (Mini-International Neuropsychiatric Interview) confirmed that the HVs did not suffer from any mental disorder and all had been free from any medication use for at least three weeks before blood sampling. The demographic data of patients and healthy volunteers are shown in Table 1.

2.3. Clinical Procedures. Psychiatrists diagnosed all the subjects while the clinical status of adolescents with MDD was determined using the validated Spanish version of the 21-item Hamilton Depression Rating Scale (HDRS) [17]. Patients had not taken any antidepressants for at least 3 weeks prior to the study. After receiving a detailed explanation of the study aims, they signed written consent forms. All patients were given SSRIs. At the screening visit, after being administered with the HRSD, every subject underwent a laboratory examination to rule out any medical illnesses. All patients were evaluated monthly throughout the study by their psychiatrist, who applied the HRSD. Figure 1 shows the total number of patients evaluated, the changes in their pharmacological treatment, and their reasons for withdrawal from the protocol.

2.4. Drugs. The dosage of fluoxetine was 20 mg/day. Doses were established for each patient by the psychiatrist and adjusted when necessary. All patients paid for their drugs out of pocket.

2.5. Serum Samples. Peripheral blood (10 mL) was collected by venipuncture from the cubital vein into Vacutainer® SST™ tubes with gel for serum separation (REF: 367988 BD Vacutainer System, Franklin Lakes, NJ, USA). Blood samples were centrifuged immediately ($1.125 \times$ g) at 4°C for 15 min to obtain serum. Serum samples were separated into aliquots and stored at −80°C until analysis.

2.6. Growth Factor Quantification. The levels of IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF were measured in serum using a Bio-Plex Pro™ Human Cytokine 27-Plex Assay kit (Lot. #5029511) per the manufacturer's instructions. Analytes were detected using streptavidin phycoerythrin and quantified in a Bio-Plex MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., CA, USA). Analyte concentrations were calculated by interpolation using standard curves in Bio-Plex Manager™ (Bio-Rad Laboratories Inc., CA, USA; version 6.1). The ranges of detection were as follows (pg/mL): IL-7: 2.9–33,292; IL-9: 0.8–9,281; IL-17A: 2.4–28,099; VEGF: 2.6–29,672; basic FGF: 1.3–14,858; G-CSF: 2.4–28,053; and GM-CSF: 1.5–17,729.

2.7. Statistical Analysis. Statistical analysis for HDRS scores and growth factors was performed using GraphPad Prism,

Table 1

(a) Demographic data

	Healthy volunteers(n = 18)	*Patients(n = 22)*
Demographics		
Age (years)	18.9 ± 1.2	17.1 ± 2.3
Gender (male/female)	4/14	4/18
BMI (kg/m^2)	23.2 ± 2.1	23.1 ± 2.1
Education (years)	12.9 ± 1.2	11.5 ± 2.6
Family history (yes/no)	3/15	8/14
First episode	NA	8
Recurrent episode	NA	14

(b) Molecular and clinimetric data

	HV	*Patients*			*Statistical post hoc analysis*		
	n = 18	*W0(n = 22)*	*W4(n = 22)*	*W8(n = 22)*	*W0 vs. HV*	*W4 vs. W0*	*W8 vs. W0*
Cytokine serum levels (pg/mL)							
IL-7	10.7 ± 1.8	23.7 ± 6.4	21.0 ± 5.5	24.3 ± 6.7	****	ns	ns
IL-9	20.7 ± 3.9	30.3 ± 7.8	28.3 ± 9.8	32.7 ± 7.8	**	ns	ns
IL-17A	348.4 ± 16.8	424.8 ± 48.5	391.1 ± 55.7	420.4 ± 36.0	****	ns	ns
FGF basic	84.8 ± 6.5	116.7 ± 18.0	98.7 ± 21.2	115.8 ± 14.2	****	**	ns
VEGF	95.7 ± 15.2	150.7 ± 33.3	146.0 ± 24.5	145.1 ± 17.5	****	ns	ns
G-CSF	232.9 ± 21.1	333.0 ± 54.5	285.3 ± 49.2	326.4 ± 45.1	****	**	ns
GM-CSF	28.3 ± 6.2	74.0 ± 17.5	57.0 ± 21.2	64.1 ± 14.2	****	**	ns
Clinical psychiatric scale							
HDRS	NA	19.41 ± 4.72	9.13 ± 3.5	6.09 ± 2.4	NA	****	****

Values are presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA with Bonferroni's post hoc. Statistical significance was attributed when $^{**}P < 0.01$ and $^{****}P < 0.0001$. HV = healthy volunteers; NA = not applicable; ns = not significant $P > 0.05$; vs. = versus; HDRS = Hamilton Depression Rating Scale; W = weeks of clinical follow-up.

version 6.00, for MAC OS X (GraphPad Software, La Jolla, CA, USA). Homogeneity of variance and normality tests were applied, followed by a one-way ANOVA with Bonferroni's post hoc test. All values were expressed as mean ± standard deviation. Statistical significance was attributed when $P < 0.05$.

3. Results

3.1. Demographic Data. Demographic data of twenty-two adolescents with MDD and eighteen healthy volunteers are described in Table 1.

3.2. HDRS. Adolescents with MDD had an HDRS score of 19.41 ± 4.72 at the beginning of the study (W0), 9.13 ± 3.5 at week four (W4), and 6.09 ± 2.4 at week eight (W8). HDRS scores at W4 and W8 were significantly lower when compared to W0, as shown in Table 1.

3.3. Growth Factors

(1) IL-7, IL-9, IL-17A, and VEGF. Serum levels of IL-7, IL-9, IL-17A, and VEGF were significantly higher in adolescents with MDD at W0 compared with those in HVs (IL-7: $F = 23.97$; IL-9: $F = 8.44$; IL-17A: $F = 13.10$; and VEGF: $F = 21.79$; in all cases $df = 80.3$ and $P < 0.0001$). Levels were consistently elevated during the eight weeks of treatment (Table 1).

(2) Basic FGF, G-CSF, and GM-CSF. We observed a significant increase in serum levels of basic FGF, G-CSF, and GM-CSF in adolescents with MDD (basic FGF: $F = 17.25$; G-CSF: $F = 20.24$; and GM-CSF: $F = 28.50$; in all cases $df = 80, 3$ and $P < 0.0001$). Interestingly, basic FGF, G-CSF, and GM-CSF showed a significant decrease after four weeks of treatment with fluoxetine (W0 vs. W4; $P < 0.01$), but they increased again at W8 (Table 1).

4. Discussion

Our results show that adolescents with MDD had a clinical improvement from week four of treatment with fluoxetine, as reported in previous works in adolescents [8, 18]. However, we found no correlation between HDRS score and the levels of growth factors (data not shown).

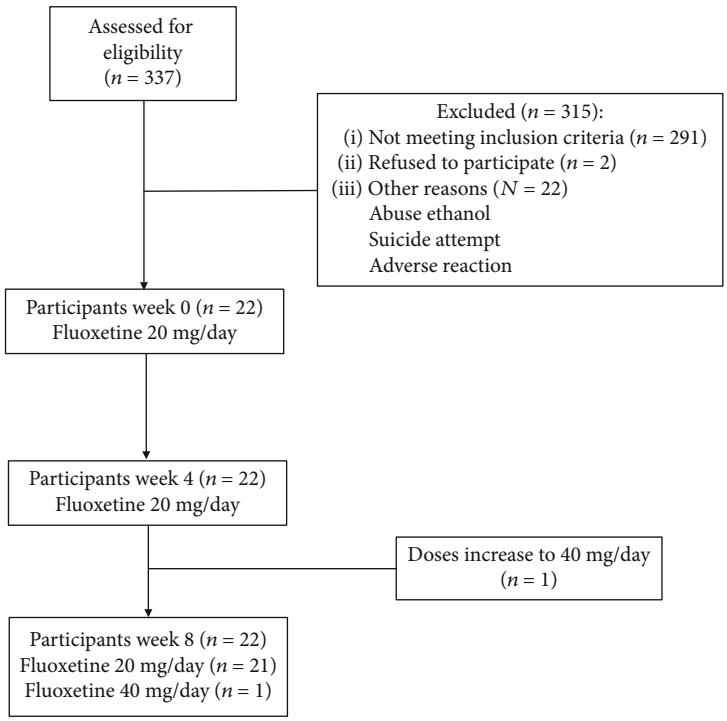

Figure 1: Flow diagram of eight-week fluoxetine treatment in adolescents with major depressive disorder.

4.1. IL-7. IL-7 is mainly associated with the development of T and B cells and is considered a necessary hematopoietic factor for the maintenance and proliferation of primary and secondary lymphoid organs [19]. Our results show significantly high levels of IL-7 in adolescents with MDD as reported in previous works [20, 21], but contrary to the report by Lehto et al. [22]. Comorbidities as sleep disorders, metabolic syndrome, number of cigarettes smoked, and daily alcohol consumption have been associated with decreased levels of IL-7 [22]. Unlike Dahl et al. [20], who reported a significant decrease of IL-7 after 12 weeks of treatment, our group found no changes in IL-7 levels during the clinical follow-up. It should be highlighted that the work of Dahl et al. was performed in adults who received dual antidepressant and SSRI treatment during 12 weeks [20]. It is known that IL-7 preferentially promotes a polarization towards Th-1, favoring a proinflammatory profile [23, 24], something common in MDD patients. Additionally, IL-7 is able to act as a neural growth factor by influencing the development of neurons at a molecular level, which in turn affects the brain architecture [25]. The link between IL-7 and neural development gains special interest when talking about MDD, and this work provides new evidence on alterations of IL-7 during the course of pharmacological treatment with fluoxetine, a topic little explored in adolescents.

4.2. IL-9. IL-9 is a Th-2 cytokine that has been associated with Th17 cells [26]. It has been widely described as a growth factor that regulates hematopoiesis, mast cell growth, and B cell development [26]. Although its role was described over 20 years ago, its direct participation in MDD is yet to be found, and there are no reports of its involvement in adolescents with MDD. Recently, Karlsson et al. detected a positive correlation between IL-9 and prenatal depression and anxiety [27] as well as a possible link between prenatal stress and the neonatal health. In addition, Shelton et al. found an increase in the expression of IL-9 in the frontal cortex of MDD patients [28], which suggests that the overexpression of IL-9 is associated with the severity of MDD. However, in our results, we did not find a correlation between IL-9 and HDRS score (data not shown).

4.3. IL-17A. IL-17A is a cytokine mainly produced by Th17 cells and is strongly associated with a series of inflammatory conditions and autoimmune diseases [29]. Moreover, it is known that IL-17 is able to stimulate angiogenesis and tumor growth [30]. In 2011, Chen et al. reported that patients with MDD coursing with an autoimmune process have high levels of IL-17 [31], but the role of IL-17 remains unclear in patients with MDD in the absence of an autoimmune disorder, as in our results. In adolescents with MDD, there is a correlation between anxiety symptoms and the levels of IL-17; moreover, the levels of this cytokine are not affected by the administration of antidepressants [7]. Our results show higher levels of IL-17 in adolescents with MDD as compared with healthy volunteers, as in previous reports in adults [7, 32], and the treatment with fluoxetine did not alter the levels of this growth factor. Interestingly, there is a study in vitro that reports that citalopram affects the levels of IL-17 in peripheral mononuclear cells from adult MDD patients [33]. Furthermore, IL-17 is mainly produced by Th17 cells, which have important roles in immune response in the CNS, as the regulation of the microglial activation. In fact, it has been suggested a role of both Th17 cells and IL-17 in the neuroprogression of MDD [34]. Moreover, in a mouse model, it was

demonstrated that the administration of IL-17 antibodies was able to produce antidepressant-like effects [35], supporting the role of IL-17 in the development of MDD.

4.4. FGF. Fibroblast growth factor (FGF) is involved in important processes as long-term potentiation, neurogenesis, and proliferation of neural progenitor cells [36], which are all closely related to MDD. It is known that FGF is altered in adults with MDD, but little is known about it in adolescents. A detailed meta-analysis on FGF showed increased levels of FGF in adults with MDD [37], which agrees with our results in adolescents. On the contrary, He et al. reported lower levels in patients with MDD as well as a reduction of FGF after eight weeks of treatment with antidepressants [12]. In this sense, we also observed a reduction of FGF during treatment, but only in a temporal manner at the fourth week. Although the role of this growth factor is associated with neurogenesis and angiogenesis, more evidence is needed to demonstrate that the peripheral levels correlate to those in the CNS. Similarly, the lack of information regarding its role in the adolescent population represents an opportunity for future studies.

4.5. VEGF. The vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis; therefore, it is considered to be a neurotrophic factor. Numerous studies in adults have shown significantly higher levels of VEGF in MDD patients versus healthy volunteers [38]. Within this frame, our results also show an increase in VEGF in adolescents with MDD but no affectations by the treatment with fluoxetine, which also agrees with previous reports [39].

4.6. G-CSF and GM-CSF. G-CSF and GM-CSF are two important hematopoietic factors that have been associated with MDD. These growth factors can promote macrophage migration from the bone marrow, giving rise to the inflammatory process observed in MDD. The link between macrophages and MDD is known as the *macrophage theory of depression* [40]. Our results in adolescents with MDD are consistent with those described in previous works in adults, which reported significantly higher levels of G-CSF and GM-CSF in depressed patients [20]. Moreover, Kiraly et al. observed a correlation between the high levels of GM-CSF in MDD patients and the subjects' resistance to antidepressants [41]. Finally, we also observed a significant but temporal reduction of G-CSF and GM-CSF at week four of treatment, as in previous reports [20].

Taken together, all these results demonstrate that GM-CSF and G-CSF may play crucial roles in MDD given that they can promote the mobilization of leukocytes from the bone marrow, leading to an increase in macrophages and the release of cytokines in inflammatory processes as depression. These findings reinforce the *macrophage theory of depression* in MDD onset [40] but now in adolescents.

5. Conclusions

Our results showed a significant increase in the circulatory levels of growth factors in adolescents with MDD in comparison with healthy volunteers (HV). This increase was consistent throughout the 8-week pharmacological treatment with fluoxetine [8]. To the best of our knowledge, this is the first report to show alterations in the levels of growth factors such as IL-7, IL-9, IL-17A, VEGF, basic FGF, G-CSF, and GM-CSF in adolescents with MDD during eight weeks of clinical follow-up. These disturbances could be involved in the physiopathology of MDD because it has been proven such growth factors participate in the neural development and correct functioning of the CNS; therefore, subtle alterations in it may contribute to MDD.

5.1. Limitations of This Study. The patient cohort was small, mainly due to the difficulties involved in pickup adolescents in clinical studies, but the numbers of HVs and patients were sufficient for statistical analysis. Also, the clinical follow-up was only 8 weeks long. In all cases, the discrepancies in our values and those by other groups can be explained by differences in samples (serum or plasma), detection techniques, sample size, age of participants, and confounding factors such as alcohol and tobacco consumption and body mass index.

Data Availability

The cytokine levels and psychiatric values used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest associated with the present manuscript.

Authors' Contributions

F DLP, C C-F, and L P designed the study, wrote the protocol, supervised the patient recruitment, collected and analyzed the data, and revised the manuscript. E B-V, G P-S, S A-H, L Palacios, R A, and I G recruited the patients, monitored their clinical responses, and supervised the collection of samples and clinimetric tests. All authors interpreted and discussed the results and contributed to and approved the final manuscript. Enrique Becerril-Villanueva and Gilberto Pérez-Sánchez wrote the manuscript and contributed equally to this work.

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References

[1] A. U. Ekwonye, T. F. Cahill, D. De Luca, and L. Cabell, "Exploring the multivariate relationships between adolescent depression and social support, religiosity, and spirituality in a faith-based high school," *Health*, vol. 9, no. 1, pp. 38–56, 2017.

[2] R. Perou, R. H. Bitsko, S. J. Blumberg et al., "Mental health surveillance among children–United States, 2005-2011," *Morbidity and Mortality Weekly Report surveillance summaries*, vol. 62, pp. 1–35, 2013.

[3] N. W. Carrellas, J. Biederman, and M. Uchida, "How prevalent and morbid are subthreshold manifestations of major depression in adolescents? A literature review," *Journal of Affective Disorders*, vol. 210, pp. 166–173, 2017.

[4] Y. Dowlati, N. Herrmann, W. Swardfager et al., "A meta-analysis of cytokines in major depression," *Biological Psychiatry*, vol. 67, no. 5, pp. 446–457, 2010.

[5] H. A. Eyre, T. Air, A. Pradhan et al., "A meta-analysis of chemokines in major depression," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 68, pp. 1–8, 2016.

[6] A. Y. Galvez-Contreras, T. Campos-Ordonez, V. Lopez-Virgen, J. Gomez-Plascencia, R. Ramos-Zuniga, and O. Gonzalez-Perez, "Growth factors as clinical biomarkers of prognosis and diagnosis in psychiatric disorders," *Cytokine and Growth Factor Reviews*, vol. 32, pp. 85–96, 2016.

[7] P. Pallavi, R. Sagar, M. Mehta et al., "Serum cytokines and anxiety in adolescent depression patients: gender effect," *Psychiatry Research*, vol. 229, no. 1-2, pp. 374–380, 2015.

[8] G. Pérez-Sánchez, E. Becerril-Villanueva, R. Arreola et al., "Inflammatory profiles in depressed adolescents treated with fluoxetine: an 8-week follow-up open study," *Mediators of Inflammation*, vol. 2018, 12 pages, 2018.

[9] M.-C. Audet and H. Anisman, "Interplay between pro-inflammatory cytokines and growth factors in depressive illnesses," *Frontiers in Cellular Neuroscience*, vol. 7, 2013.

[10] H. B. Stolp, "Neuropoietic cytokines in normal brain development and neurodevelopmental disorders," *Molecular and Cellular Neurosciences*, vol. 53, pp. 63–68, 2013.

[11] D. Berent, M. Macander, J. Szemraj, A. Orzechowska, and P. Gałecki, "Vascular endothelial growth factor a gene expression level is higher in patients with major depressive disorder and not affected by cigarette smoking, hyperlipidemia or treatment with statins," *Acta Neurobiologiae Experimentalis*, vol. 74, no. 1, pp. 82–90, 2014.

[12] S. He, T. Zhang, B. Hong et al., "Decreased serum fibroblast growth factor - 2 levels in pre- and post-treatment patients with major depressive disorder," *Neuroscience Letters*, vol. 579, pp. 168–172, 2014.

[13] B. H. Lee and Y. K. Kim, "The roles of BDNF in the pathophysiology of major depression and in antidepressant treatment," *Psychiatry Investigation*, vol. 7, no. 4, pp. 231–235, 2010.

[14] M. Martino, G. Rocchi, A. Escelsior et al., "NGF serum levels variations in major depressed patients receiving duloxetine," *Psychoneuroendocrinology*, vol. 38, no. 9, pp. 1824–1828, 2013.

[15] E. Milanesi, A. Minelli, N. Cattane et al., "ErbB3 mRNA leukocyte levels as a biomarker for major depressive disorder," *BMC Psychiatry*, vol. 12, no. 1, article 145, 2012.

[16] A. N. Sharma, B. F. Borges da Costa e Silva, J. C. Soares, A. F. Carvalho, and J. Quevedo, "Role of trophic factors GDNF, IGF-1 and VEGF in major depressive disorder: a comprehensive review of human studies," *Journal of Affective Disorders*, vol. 197, pp. 9–20, 2016.

[17] M. C. Hamilton, "5.2. Hamilton depression scale (HDS) with melancholia scale (MES)," *Acta Psychiatrica Scandinavica*, vol. 73, no. S326, pp. 23–28, 1960.

[18] P. Gassó, N. Rodríguez, D. Boloc et al., "Association of regulatory *TPH2* polymorphisms with higher reduction in depressive symptoms in children and adolescents treated with fluoxetine," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 77, pp. 236–240, 2017.

[19] L. Onder, P. Narang, E. Scandella et al., "IL-7-producing stromal cells are critical for lymph node remodeling," *Blood*, vol. 120, no. 24, pp. 4675–4683, 2012.

[20] J. Dahl, H. Ormstad, H. C. D. Aass et al., "The plasma levels of various cytokines are increased during ongoing depression and are reduced to normal levels after recovery," *Psychoneuroendocrinology*, vol. 45, pp. 77–86, 2014.

[21] N. M. Simon, K. McNamara, C. W. Chow et al., "A detailed examination of cytokine abnormalities in major depressive disorder," *European Neuropsychopharmacology*, vol. 18, no. 3, pp. 230–233, 2008.

[22] S. M. Lehto, A. Huotari, L. Niskanen et al., "Serum IL-7 and G-CSF in major depressive disorder," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 34, no. 6, pp. 846–851, 2010.

[23] S. I. Gringhuis, L. F. M. H. de Leij, E. W. Verschuren, P. Borger, and E. Vellenga, "Interleukin-7 upregulates the interleukin-2–gene expression in activated human T lymphocytes at the transcriptional level by enhancing the DNA binding activities of both nuclear factor of activated T cells and activator protein-1," *Blood*, vol. 90, pp. 2690–2700, 1997.

[24] A. H. Miller, "Depression and immunity: a role for T cells?," *Brain, Behavior, and Immunity*, vol. 24, no. 1, pp. 1–8, 2010.

[25] M. Moors, N. K. Vudattu, J. Abel et al., "Interleukin-7 (IL-7) and IL-7 splice variants affect differentiation of human neural progenitor cells," *Genes and Immunity*, vol. 11, no. 1, pp. 11–20, 2010.

[26] R. Goswami, "Th9 cells: new member of T helper cell family," in *Th9 Cells*, R. Goswami, Ed., vol. 1585 of Methods in Molecular Biology, pp. 1–19, Humana Press, New York, NY, USA, 2017.

[27] L. Karlsson, N. Nousiainen, N. M. Scheinin et al., "Cytokine profile and maternal depression and anxiety symptoms in mid-pregnancy—the FinnBrain birth cohort study," *Archives of Women's Mental Health*, vol. 20, no. 1, pp. 39–48, 2017.

[28] R. C. Shelton, J. Claiborne, M. Sidoryk-Wegrzynowicz et al., "Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression," *Molecular Psychiatry*, vol. 16, no. 7, pp. 751–762, 2011.

[29] S. Zhu and Y. Qian, "IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential," *Clinical Science*, vol. 122, no. 11, pp. 487–511, 2012.

[30] M. Numasaki, J. I. Fukushi, M. Ono et al., "Interleukin-17 promotes angiogenesis and tumor growth," *Blood*, vol. 101, no. 7, pp. 2620–2627, 2003.

[31] Y. Chen, T. Jiang, P. Chen et al., "Emerging tendency towards autoimmune process in major depressive patients: a novel insight from Th17 cells," *Psychiatry Research*, vol. 188, no. 2, pp. 224–230, 2011.

[32] J.-W. Kim, Y.-K. Kim, J.-A. Hwang et al., "Plasma levels of IL-23 and IL-17 before and after antidepressant treatment in patients with major depressive disorder," *Psychiatry Investigation*, vol. 10, no. 3, pp. 294–299, 2013.

[33] A. Munzer, U. Sack, R. Mergl et al., "Impact of antidepressants on cytokine production of depressed patients in vitro," *Toxins*, vol. 5, no. 11, pp. 2227–2240, 2013.

[34] A. Slyepchenko, M. Maes, C. A. Köhler et al., "T helper 17 cells may drive neuroprogression in major depressive disorder: proposal of an integrative model," *Neuroscience and Biobehavioral Reviews*, vol. 64, pp. 83–100, 2016.

[35] E. Beurel, L. E. Harrington, and R. S. Jope, "Inflammatory T helper 17 cells promote depression-like behavior in mice," *Biological Psychiatry*, vol. 73, no. 7, pp. 622–630, 2013.

[36] M. Zhao, D. Li, K. Shimazu, Y. X. Zhou, B. Lu, and C. X. Deng, "Fibroblast growth factor receptor-1 is required for long-term potentiation, memory consolidation, and neurogenesis," *Biological Psychiatry*, vol. 62, no. 5, pp. 381–390, 2007.

[37] C. Wu, P. Tseng, Y. Chen, K. Tu, and P. Lin, "Significantly higher peripheral fibroblast growth factor-2 levels in patients with major depressive disorder: a preliminary meta-analysis under MOOSE guidelines," *Medicine*, vol. 95, article e4563, 2016.

[38] P.-T. Tseng, Y.-S. Cheng, Y.-W. Chen, C.-K. Wu, and P.-Y. Lin, "Increased levels of vascular endothelial growth factor in patients with major depressive disorder: a meta-analysis," *European Neuropsychopharmacology*, vol. 25, no. 10, pp. 1622–1630, 2015.

[39] A. Clark-Raymond, E. Meresh, D. Hoppensteadt et al., "Vascular endothelial growth factor: potential predictor of treatment response in major depression," *The World Journal of Biological Psychiatry*, vol. 18, pp. 575–585, 2017.

[40] R. S. Smith, "The macrophage theory of depression," *Medical Hypotheses*, vol. 35, no. 4, pp. 298–306, 1991.

[41] D. D. Kiraly, S. R. Horn, N. T. Van Dam et al., "Altered peripheral immune profiles in treatment-resistant depression: response to ketamine and prediction of treatment outcome," *Translational Psychiatry*, vol. 7, no. 3, article e1065, 2017.

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Research Article

METTL3 Attenuates LPS-Induced Inflammatory Response in Macrophages via NF-κB Signaling Pathway

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Methyltransferase-like 3 (METTL3), an RNA N^6-methyladenosine (m^6A) methyltransferase, is essential for the m^6A mRNA modification. As a key enzyme of m^6A methylation modification, METTL3 has been implicated in immune and inflammation regulation. However, little is known of the role and underlying mechanism of METTL3 in rheumatoid arthritis (RA). The aim of the present study is to elucidate the function and potential mechanism of METTL3 in RA pathogenesis. We used quantitative real-time polymerase chain reaction to detect the expression of METTL3 in RA patients and controls as well as the macrophage cell line. CCK-8 was used for cell proliferation assay. Enzyme-linked immunosorbent assay (ELISA) was adopted to estimate the generation of IL-6 and TNF-α in macrophages. Western blot and immunofluorescence were applied to evaluate the activation of NF-κB in macrophages. The expression of METTL3 was significantly elevated in patients with RA. It was positively associated with CRP and ESR, two common markers for RA disease activity. Besides, LPS could enhance the expression and biological activity of METTL3 in macrophages, while overexpression of METTL3 significantly attenuated the inflammatory response induced by LPS in macrophages. Moreover, the effect of METTL3 on LPS-induced inflammation in macrophages was dependent on NF-κB. This study firstly demonstrates the critical role of METTL3 in RA, which provides novel insights into recognizing the pathogenesis of RA and a promising biomarker for RA.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with chronic and reduplicated joint destruction, which is a highly disabling disease because of joint deformity and loss of function [1]. Joint inflammation can cause redness, swelling, pain, and joint deformity [2]. The etiology of RA is still largely unknown. It has been well established that the development of RA is attributed to genetic and environmental factors, such as tobacco smoking [3]. Apart from those, obesity, stress, nervous depression, and female hormones play vital roles in the pathogenesis of RA [3]. Many studies have suggested that dysregulation of the immune system, including abnormal activation and T and B lymphocytes, mast cells, and macrophages, is closely associated with the development of RA [4]. High levels of autoantibodies generated by dysregulated B cells can cause lung injuries, such as anticitrullinated protein antibodies [5, 6]. Inflammatory cells can be attracted and recruited to inflammatory sites under stimulation of considerable mediators from macrophages and mast cells. Thus, sustained and chronic inflammation leads to joint injuries and deformity [1]. As a result, it is essential to identify novel and promising biomarkers for the early diagnosis and targeted therapy of RA patients.

During the past few years, the role of N^6-methyladenosine (m^6A) methylation of RNA in autoimmunity, inflammation,

Table 1: Characteristics of participants.

	RA ($n = 47$)	Healthy controls ($n = 30$)	P value
Age (yrs, mean ± SD)	58.3 ± 6.1	49.9 ± 8.7	0.242
Gender (females/males)	34/13	21/9	0.105
Disease course (years)	16.6 ± 5.2	—	—
CRP (mg/l)	20.4 ± 3.5	—	—
ESR (mm/h)	41.0 ± 7.5	—	—
RF (IU/ml)	191.6 ± 5.7	—	—

and cancer has drawn close attention [7–10]. m^6A is the most common posttranscription modification of RNA [11]. The key enzymes for m^6A methylation modification primarily include m^6A methyltransferase (writer), m^6A demethylase (eraser), and m^6A RNA-binding proteins (reader) [12]. Methyltransferase-like 3 (METTL3) is a key enzyme of m^6A methylation modification and an important member of the methyltransferase complex including METTL3, METTL4, and Wilms tumor 1-associated protein (WTAP) [13]. It has been demonstrated that m^6A methylation mediated by METTL3 has tissue and cell specificity [12–15]. Therefore, the role of METTL3 may alter in different tissues and cells [16]. METTL3 has been previously reported as a tumor suppressor by upregulating the m6A modification of genes [17]. However, Chen and colleagues have found that METTL3 could promote liver cancer progression by posttranscriptional silencing of SOCS2 via YTHDF2 [18]. More interestingly, the study by Feng et al. has implicated that METTL3 could inhibit inflammation by affecting the alternative splicing of MyD88 [19]. Nevertheless, the role of METTL3 in RA, an autoimmune and inflammatory disease, remains vague up to date. In the current study, we investigate the expression of METTL3 in RA and its relationship with disease activity. A series of cellular experiments in vitro have been performed to elucidate the potential role of METTL3 and its molecular mechanisms in RA, which may help to explore the pathogenesis of RA and explore novel biomarkers for RA.

2. Materials and Methods

2.1. Participants. Patients (47) who participated in this study were admitted to the affiliated hospital of Weifang Medical University from March 2018 to September 2018. 30 controls registered in the same hospital at the same time for health examination, while controls with a history of rheumatoid diseases were all excluded. All RA cases were new active patients and had not yet been treated with disease-modifying antirheumatic drugs and/or steroids before blood sample collection. Those RA patients accompanied by other rheumatoid diseases were excluded, such as Sjogren's disease. The present study was approved by the Institutional Ethics Committee of our hospital. Patients and controls had all signed the written informed consent before tests. Characteristics of all participants are presented in detail in Table 1.

2.2. Cell Culture and Transfection. THP-1 cells were cultured in RPMI1640 culture medium (Invitrogen Corp., Grand Island, NYUSA) administrated with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) plus penicillin, streptomycin, and L-glutamine under 5% CO_2 at 37°C. Before treatment, THP-1 cells were stimulated and induced to macrophage-like cells (pTHP-1) by 100 ng/ml phorbol-12-myristate-13 acetate (PMA, Sigma, USA) for 48 h. Cells were transfected by lentivirus plasmid with polybrene (8 μg/ml) for 48 h and used for the following experiments.

2.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Peripheral blood mononuclear cells (PBMCs) were extracted from fresh blood samples by Ficoll-Paque lymphocyte isolate reagent (TBD, Tianjin, China), which were centrifuged at 2000 rpm for 30 min. We extracted peripheral blood monocytes using CD14 microbeads (Miltenyi Biotec, San Diego, CA) based on the protocol. Total RNAs were extracted from these cells by TRIzol (Invitrogen, USA) and quantified by measuring the absorbance with the UV spectrophotometer. A total of 1 μg RNA was used to synthesize cDNA, which was used as a template for PCR by use of a SYBR Green Mastermix kit (Takara, Dalian, China). Primers were shown as follows. GAPDH: forward primer: GCACCGTCAAGGCTGAGAAC; reverse primer: GGAT CTCGCTCCTGGAAGATG. METTL3: forward primer: TTGTCTCCAACCTTCCGTAGT; reverse primer: CCAG ATCAGAGAGGTGGTGTAG. METTL14: forward primer: AGTGCCGACAGCATTGGTG; reverse primer: GGAGCA GAGGTATCATAGGAAGC. Obesity-associated protein (FTO): forward primer: AGAGCTCTAGAACCACCATGG ATTACAAAGATGAC; reverse primer: CTAAGATTGCG GCCGCCTAGGGTTTTGCTTCCAGAAGC. AlkB homologue 5 (ALKBH5): forward primer: CGGCGAAGGCT ACACTTACG; reverse primer: CCACCAGCTTTTGGAT CACCA. YTHGF1: forward primer: ACCTGTCCAGCTAT TACCCG; reverse primer: TGGTGAGGTATGGAATCGG AG. YTHGF2: forward primer: AGCCCCACTTCCTACC AGATG; reverse primer: TGAGAACTGTTATTTCCCCAT GC. IL-6: forward primer: AGTCCTGATCCAGTTCCTGC; reverse primer: CTACATTTGCCGAAGAGCCC. TNF-α: forward primer: ATGTGGCAAGAGATGGGGAA; reverse primer: CTCACACCCCACATCTGTCT.

2.4. Total m^6A Measurement. Cells were seeded in six-well culture dishes overnight in serum-free RPMI1640 culture medium. Then, cells were treated with 1 μg/ml LPS (Sigma, CA, USA) for 0, 6, 12, and 24 hrs. RNAs were extracted according to the protocol. The kit for methylation quantification (EpiGentek, Farmingdale, NY, USA) was applied to determine levels of m^6A RNA modification in cells.

2.5. Cell Counting Kit (CCK-8). We used CCK-8 kit (Vazyme Biotech, Nanjing, China) to determine cell proliferation. In brief, 2×10^4 per well THP-1 cells were incubated and activated by PMA (100 ng/ml) for 48 hrs in a 96-well plate and cultured with serum-free culture medium for 12 hrs and then stimulated by 1 μg/ml LPS (Sigma, USA) for 0, 12, 24, and 48 hrs. The absorption at 450 nm was determined by the

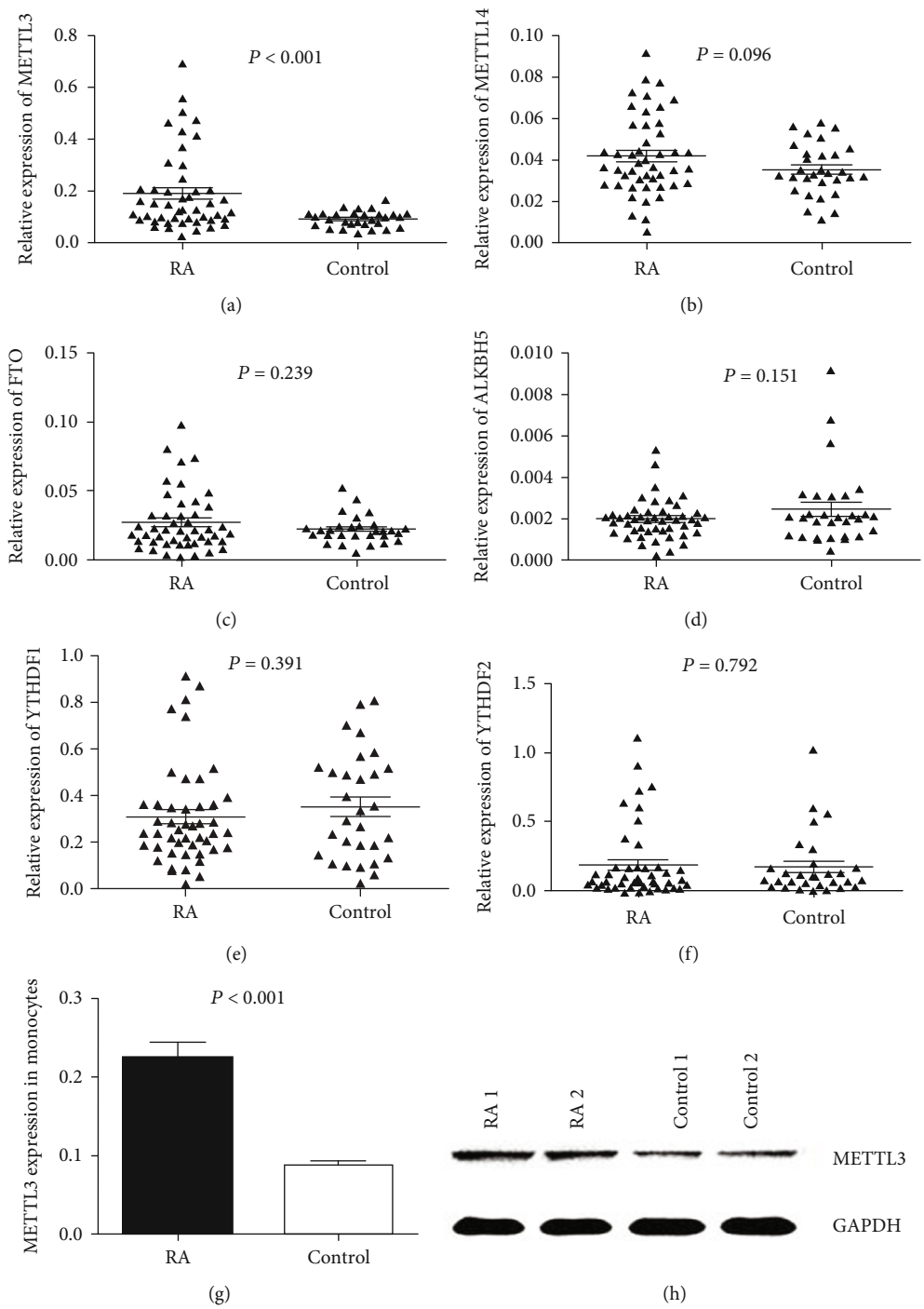

Figure 1: Expression of m^{6}A methylation-related genes in RA (cases/controls: 47/30). (a) Increased mRNA level of METTL3 in RA in contrast to healthy controls. (b) mRNA level of METTL14 in RA when comparing with healthy controls. (c) mRNA level of FTO in RA compared with healthy controls. (d) mRNA level of ALKBH5 in RA in contrast to healthy controls. (e) mRNA level of YTHDF1 in RA when comparing with healthy controls. (f) mRNA level of YTHDF2 in RA in contrast to healthy controls. (g) Increased mRNA level of METTL3 in monocytes of RA patients in contrast to controls. (h) Increased METTL3 protein in monocytes of RA patients in contrast to controls.

microplate reader (BioTek, USA) after incubation with CCK-8 reagent for 2 hrs at 37°C.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). The concentrations of IL-6, TNF-α, ESR, and CRP were determined by ELISA according to the kit's protocol (R&D Systems, USA). We determined the absorption at the wavelength of 450 nm with a correction wavelength of 540 nm. Experiments were repeated for three times.

2.7. Western Blot. A total of 30 μg/channel proteins were used for detection extracted from peripheral blood monocytes and pTHP-1 cells. We adopted the Bradford assay kit (Bio-Rad Laboratories, CA, USA) to quantify proteins. Protein samples

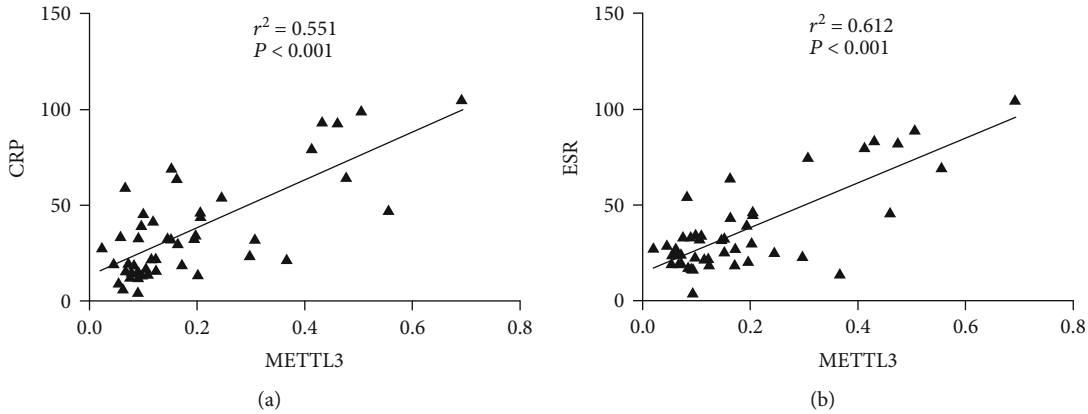

Figure 2: Association between METTL3 and RA disease activity. (a) Positive association of METTL3 with CRP in RA. (b) Positive association of METTL3 with ESR in RA.

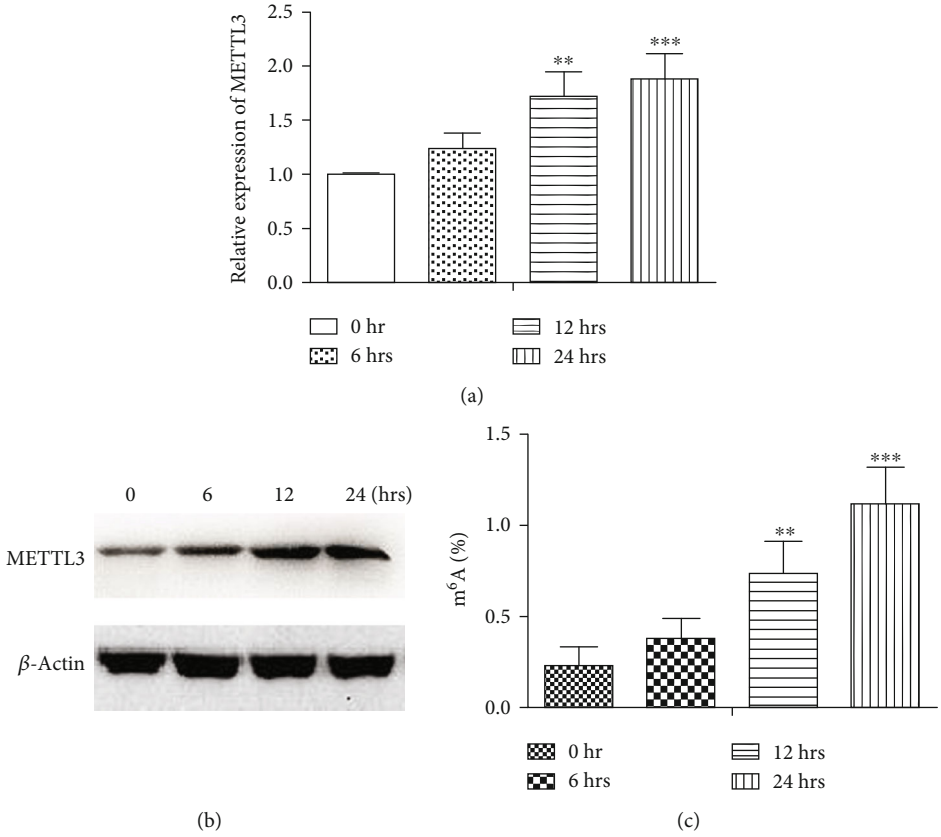

Figure 3: LPS promoted the expression and activity of METTL3 in pTHP-1 cells. (a) Increased mRNA level of METTL3 in cells ($n = 3$; LPS stimulation for 0 hr, 6 hrs, 12 hrs, and 24 hrs; $^{**}P < 0.01$ and $^{***}P < 0.001$). (b) Increased expression of METTL3 protein in cells ($n = 3$; LPS stimulation for 0 hr, 6 hrs, 12 hrs, and 24 hrs). (c) Total m^6A content in cells (LPS stimulation for 0 hr, 6 hrs, 12 hrs, and 24 hrs; $n = 3$; $^{**}P < 0.01$ and $^{***}P < 0.001$).

were incubated with antibodies of METTL3 (Cell Signaling Technology, USA) and p-NF-κB (Cell Signaling Technology, USA), and β-actin (Sigma, USA).

2.8. Immunofluorescence. After being activated by LPS (1 μg/ml) for 4 hrs, the status of phosphorylated NF-κB in the nuclear of pTHP-1 cells was estimated by immunofluorescence. Briefly, pTHP-1 cells were incubated with p-NF-κB antibody (CST, USA) and then the secondary antibodies labelled with FITC. Finally, cells were detected using a Confocal Laser Scanning Microscope (Leica TCS SP8).

2.9. Statistical Analysis. Statistical analysis was carried out by use of GraphPad Prism (GraphPad Software, CA, USA). Data used for analysis was normal distributed. Unpaired Student's t-test or one-way ANOVA was used for statistical analysis.

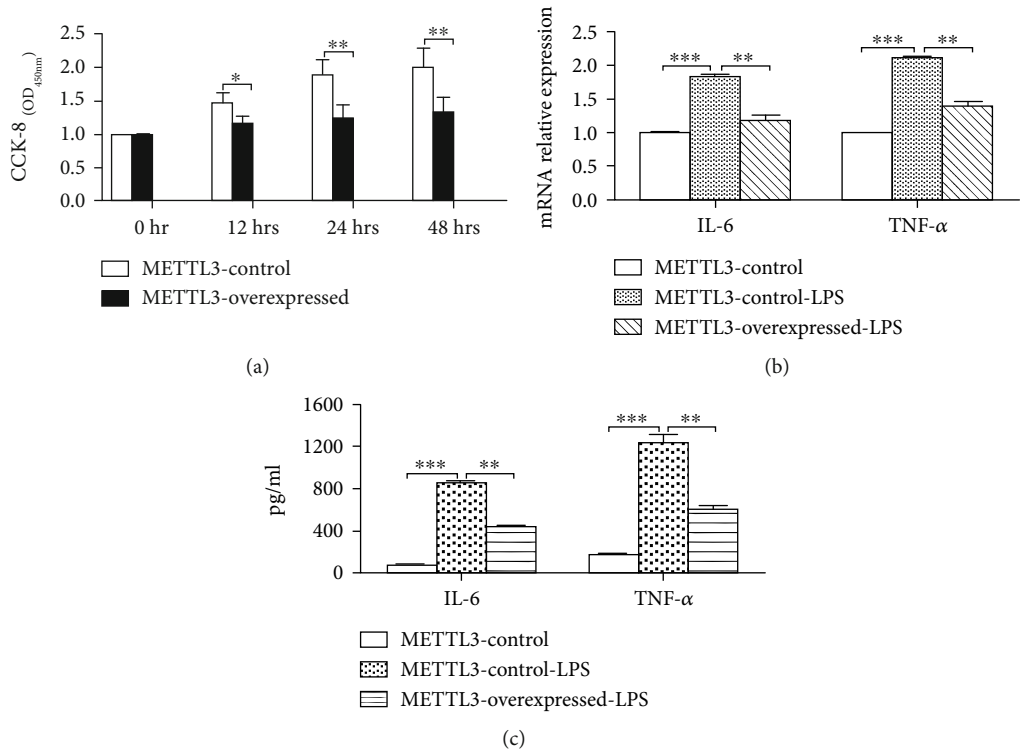

Figure 4: METTL3 inhibited the proliferation and activation of pTHP-1 cells. (a) CCK-8 assay detecting the proliferation of cells at 0 hr, 12 hrs, 24 hrs, and 48 hrs ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$). (b) Decreased mRNA levels of IL-6 and TNF-α in METTL3-overexpressed pTHP-1 cells ($n = 3$; $^{**}P < 0.01$; $^{***}P < 0.001$). (c) Decreased levels of IL-6 and TNF-α protein in the cultural supernatant of METTL3-overexpressed cells ($n = 3$; $^{**}P < 0.01$ and $^{***}P < 0.001$).

Pearson correlation analysis was used when analyzing the association between the expression of METTL3 and levels of ESR and CRP in serum. Two-tailed $P < 0.05$ was regarded to be statistically significant.

3. Results

3.1. Expression of RNA N6 Methylation-Related Genes in RA. We screened the expression of m^6A methylation-related genes in the PBMCs of patients with RA and healthy controls by quantitative real-time PCR, including genes of METTL3, METTL14, FTO, ALKBH5, YTHDF1, and YTHDF2. As shown in Figure 1(a)–1(f), compared with normal controls, the expression of METTL3 was significantly increased in PBMCs from RA patients, while no difference was observed with regard to other key m^6A methylation-related enzymes (METTL14, FTO, ALKBH5, YTHDF1, and YTHDF2). Monocytes were the main cells involved in inflammation and immune regulations. Here, elevated expression of METTL3 was also found in monocytes of RA patients in contrast to controls (Figures 1(g) and 1(h)). Taken together, METTL3 was upregulated in RA.

3.2. Association between METTL3 and Disease Activity of RA Patients. Interestingly, Pearson correlation analysis showed that the expression of METTL3 was positively associated with CRP (Figure 2(a)). Similarly, positive association of the expression of METTL3 with ESR was observed in RA (Figure 2(b)). Accordingly, the elevated level of METTL3 in PBMCs might predict high disease activity of patients with RA.

3.3. LPS Enhanced the Expression of METTL3. Given the positive association between METTL3 and CRP as well as ESR in RA, we hypothesized that a high level of METTL3 could be induced in inflammatory conditions, which could thus defend against inflammation. As a result, we performed cellular experiments in vitro to observe the influence of inflammation on METTL3 expression and the level of m^6A RNA modification in pTHP-1 macrophages. LPS could enhance the expression of METTL3 at both levels of mRNA and protein in a time-dependent manner (Figures 3(a) and 3(b)). Levels of m^6A RNA modification were also increased in pTHP-1 macrophages in a time-dependent manner (Figure 3(c)). Taken together, inflammation could promote the expression and biological activity of METTL3. However, whether METTL3 affected inflammation in macrophages and RA development remained unknown.

3.4. METTL3 Inhibited the Activation of pTHP-1 Macrophages. As evidenced by the CCK-8 assay, the proliferation of macrophages was significantly inhibited in METTL3-overexpressed pTHP-1 cells after being activated by LPS for 12, 24, and 48 hrs (Figure 4(a)). In addition, the generation of IL-6 and TNF-α induced by LPS was obviously prevented when METTL3 was overexpressed in pTHP-1

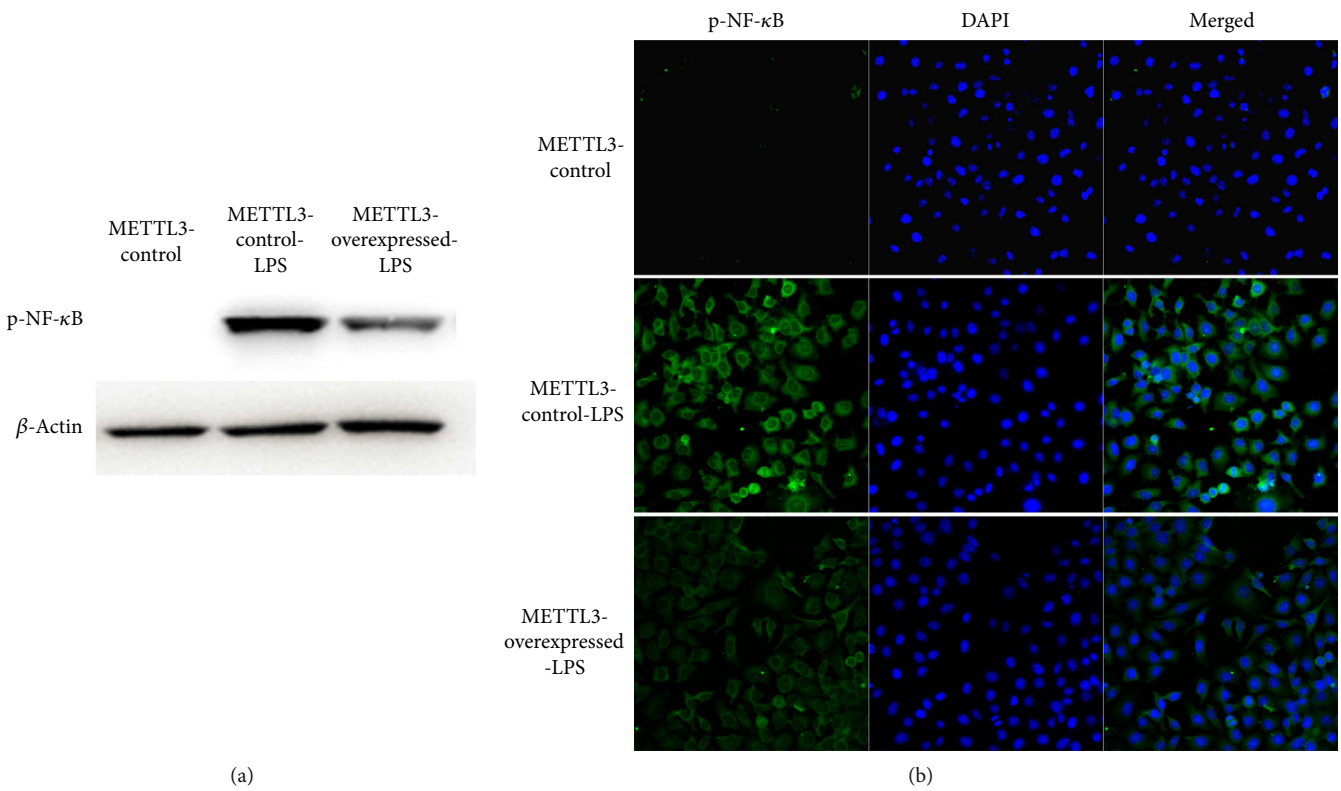

Figure 5: METTL3 attenuated LPS-induced inflammation in macrophages through the NF-κB signaling pathway. (a) METTL3 inhibited the phosphorylation of NF-κB in cells induced by LPS ($n = 3$). (b) METTL3 inhibited the nucleus translocation of p-NF-κB in cells induced by LPS ($n = 3$).

macrophages (Figures 4(b) and 4(c)). Accordingly, as a key enzyme of N^6-methyladenosine (m^6A) methylation, METTL3 could affect RA by inhibiting the proliferation and inflammatory response in macrophages, which played a crucial role in RA.

3.5. METTL3 Inhibited the Inflammatory Response of pTHP-1 Macrophages through NF-κB. Dysregulation of METTL3 seemed to influence the inflammation response of macrophages. Nonetheless, the potential molecular mechanism remained vague. In this study, we found that the phosphorylation of NF-κB was obviously restrained in METTL3-overexpressed pTHP-1 cells although cells were activated by LPS for 4 hrs (Figure 5(a)). Besides, the nuclear translocation of phosphorylated NF-κB in cells was also restrained when METTL3 was overexpressed (Figure 5(b)). Taken together, the modifying effects of METTL3 on LPS-induced inflammation in macrophages were dependent on the transcriptional factor NF-κB.

4. Discussion

RA is a chronic and systemic autoimmune disease, the etiology of which is largely unknown [20]. It has been well documented that multiple factors are associated with inflammation and immune disorders in RA, including genetic factors, environmental factors, and epigenetic deregulation [21, 22]. Various inflammatory mediators are responsible for arthritis and articular deformity, such as TNF-α, IL-6, and IL-17 [23]. Apart from inflammation, autoimmune disorders are closely related to the development and progression of RA. As a key enzyme of m^6A methylation modification, METTL3 has been suggested to regulate inflammation and autoimmune balance [13, 19]. The present study firstly shows the evidence that METTL3 is upregulated in RA and positively associated with the disease activity. Besides, METTL3 inhibits the proliferation and activation of macrophages through NF-κB.

m^6A is a methylation at the N6 position of adenosine, which is regarded as the most abundant epitranscriptomic modification of mRNA in eukaryotic cells [24]. A number of studies have demonstrated that m^6A methylation is involved in various physiological processes including mRNA stability and translation. Its modifying effects on embryonic development, cell differentiation, and stress have been well demonstrated [24–26]. There are several key genes involved in m^6A methylation modification, primarily including METTL3, METTL14, FTO, ALKBH5, and YTHDF [27, 28]. METTL3 is also known as MTA70, which is originally identified as a methyltransferase involved in the process of m^6A methylation [29]. Accumulated evidence has strongly supported that METTL3 is involved in a variety of physiological contexts and in cancers [30–32]. METTL3 is located in a nuclear speckle, which reveals that METTL3 may play an important role in RNA metabolism [33, 34]. Given its effects on inflammation, cancer, and immune regulation [19], we hypothesize that METTL3 may affect the development and progression of RA by regulating

macrophage-mediated inflammation. In this study, we firstly detected the expression of m^6A methylation-associated genes (METTL3, FTO, ALKBH5, METTL14, YTHDF1, and YTHDF2) in PBMCs from RA patients and found that METTL3 was obviously upregulated in RA compared with healthy controls. Interestingly, LPS stimulation could enhance total m^6A content of macrophages in a time-dependent manner by upregulating METTL3. It can be concluded that METTL3 is upregulated in macrophages under the circumstances of inflammation.

Macrophages are activated when they are infected by pathogenic factors such as LPS, PGN, and nucleotide compositions of pathogenic microorganisms, which leads to the phosphorylation and translocation of the transcriptional factor NF-κB to the nucleus and induces the generation of target genes related to inflammation, for instance, IL-6 and TNF-α [33, 35, 36]. The NF-κB signaling pathway is a classic pathway related to inflammation and immune regulation in RA [37]. Growing data showed that expression and regulation of m^6A methylation-related genes are associated with a variety of immune signaling pathways, particularly the NF-κB pathway [8, 18]. In this study, METTL3 has been found to be upregulated in peripheral blood monocytes, the critical cell type in PMBCs involved in inflammation and immune regulations. In addition, we have demonstrated the effect of METTL3 on m^6A modification in macrophages stimulated by LPS. As a result, we hypothesize that METTL3-dependent m^6A modification is associated with inflammation induced by LPS in macrophages. After verification in experiments in vitro, we have found that METTL3 could prevent macrophages from proliferation and production of inflammation-associated cytokines, namely, IL-6 and TNF-α. Moreover, its inhibitory effects on LPS-induced inflammation in macrophages were dependent on the NF-κB signaling pathway. However, whether other key genes of this typical signaling pathway are involved in m^6A modification in macrophages except for NF-κB needs to be investigated by more future studies.

In summary, this study shows strong evidence supporting the vital role of METTL3 in RA. METTL3 can attenuate LPS-induced inflammation in macrophages through NF-κB. The findings in this study are useful for understanding RA pathogenesis and exploring novel biomarkers for RA diagnosis and treatment.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Jinghua Wang, Shushan Yan, and Hongying Lu are co-first authors.

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References

[1] D. L. Scott, F. Wolfe, and T. W. Huizinga, "Rheumatoid arthritis," *Lancet*, vol. 376, no. 9746, pp. 1094–1108, 2010.

[2] D. Aletaha, T. Neogi, A. J. Silman et al., "2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative," *Arthritis and Rheumatism*, vol. 62, no. 9, pp. 2569–2581, 2010.

[3] W. M. Jefferies, "The etiology of rheumatoid arthritis," *Medical Hypotheses*, vol. 51, no. 2, pp. 111–114, 1998.

[4] L. Klareskog, K. Lundberg, and V. Malmstrom, "Autoimmunity in rheumatoid arthritis: citrulline immunity and beyond," *Advances in immunology*, vol. 118, pp. 129–158, 2013.

[5] J. Suurmond, A. J. Schuerwegh, and R. E. Toes, "Anti-citrullinated protein antibodies in rheumatoid arthritis: a functional role for mast cells and basophils?," *Annals of the Rheumatic Diseases*, vol. 70, pp. 55–58, 2011.

[6] E. Perry, C. Kelly, P. Eggleton, A. De Soyza, and D. Hutchinson, "The lung in ACPA-positive rheumatoid arthritis: an initiating site of injury?," *Rheumatology*, vol. 53, no. 11, pp. 1940–1950, 2014.

[7] X. Wu, L. Sang, and Y. Gong, "N6-Methyladenine RNA modification and cancers," *American journal of Cancer Research*, vol. 8, no. 10, pp. 1957–1966, 2018.

[8] K. Hong, "Emerging function of N6-methyladenosine in cancer," *Oncology Letters*, vol. 16, no. 5, pp. 5519–5524, 2018.

[9] R. M. Rubio, D. P. Depledge, C. Bianco et al., "RNA m(6)A modification enzymes shape innate responses to DNA by regulating interferon beta," *Genes & Development*, vol. 32, no. 23-24, pp. 1472–1484, 2018.

[10] N. Lu, X. Li, J. Yu et al., "Curcumin attenuates lipopolysaccharide-induced hepatic lipid metabolism disorder by modification of m(6) A RNA methylation in piglets," *Lipids*, vol. 53, no. 1, pp. 53–63, 2018.

[11] X. Yin, R. Sachidanandam, S. Morshed, R. Latif, R. Shi, and T. F. Davies, "mRNA-Seq reveals novel molecular mechanisms and a robust fingerprint in Graves' disease," *The Journal of Clinical Endocrinology and Metabolism*, vol. 99, no. 10, pp. E2076–E2083, 2014.

[12] W. V. Gilbert and T. A. Bell, "Messenger RNA modifications: form, distribution, and function," *Science*, vol. 352, pp. 1408–1412, 2016.

[13] S. Geula, S. Moshitch-Moshkovitz, D. Dominissini et al., "Stem cells. m6A mRNA methylation facilitates resolution of naive pluripotency toward differentiation," *Science*, vol. 347, no. 6225, pp. 1002–1006, 2015.

[14] K. D. Meyer, "Rethinking m6A readers, writers, and erasers," *Annual Review of Cell and Development Biology*, vol. 33, pp. 319–342, 2017.

[15] S. Geula, S. Moshitch-Moshkovitz, D. Dominissini et al., "Stem cells m6A mRNA methylation facilitates resolution of naïve

pluripotency toward differentiation," *Science*, vol. 347, no. 6225, pp. 1002–1006, 2015.

[16] J. Liu, M. A. Eckert, B. T. Harada et al., "m(6)A mRNA methylation regulates AKT activity to promote the proliferation and tumorigenicity of endometrial cancer," *Nature Cell Biology*, vol. 20, no. 9, pp. 1074–1083, 2018.

[17] L. He, J. Li, X. Wang et al., "The dual role of N6-methyladenosine modification of RNAs is involved in human cancers," *Journal of Cellular and Molecular Medicine*, vol. 22, no. 10, pp. 4630–4639, 2018.

[18] M. Chen, L. Wei, C. T. Law et al., "RNA N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through YTHDF2-dependent posttranscriptional silencing of SOCS2," *Hepatology*, vol. 67, no. 6, pp. 2254–2270, 2018.

[19] Z. Feng, Q. Li, R. Meng, B. Yi, and Q. Xu, "METTL3 regulates alternative splicing of MyD88 upon the lipopolysaccharide-induced inflammatory response in human dental pulp cells," *Journal of Cellular and Molecular Medicine*, vol. 22, no. 5, pp. 2558–2568, 2018.

[20] F. R, L. R, and S. S. J, "Review of head-to-head study designs in rheumatoid arthritis," *Seminars in Arthritis and Rheumatism*, vol. 46, no. 3, pp. 279–285, 2016.

[21] T. T. Glant, K. Mikecz, and T. A. Rauch, "Epigenetics in the pathogenesis of rheumatoid arthritis," *BMC Medicine*, vol. 12, p. 35, 2014.

[22] E. Karouzakis, R. E. Gay, S. Gay, and M. Neidhart, "Epigenetic deregulation in rheumatoid arthritis," *Epigenetic Contributions in Autoimmune Disease*, vol. 711, pp. 137–149, 2011.

[23] F. M. Brennan and I. B. McInnes, "Evidence that cytokines play a role in rheumatoid arthritis," *The Journal of Clinical Investigation*, vol. 118, no. 11, pp. 3537–3545, 2008.

[24] J. M. Adams and S. Cory, "Modified nucleosides and bizarre 5′-termini in mouse myeloma mRNA," *Nature*, vol. 255, no. 5503, pp. 28–33, 1975.

[25] Y. Furuichi, M. Morgan, A. J. Shatkin, W. Jelinek, M. Salditt-Georgieff, and J. E. Darnell, "Methylated, blocked 5 termini in HeLa cell mRNA," *Proceedings of the National Academy of Sciences*, vol. 72, no. 5, pp. 1904–1908, 1975.

[26] Y. Yang, P. J. Hsu, Y. S. Chen, and Y. G. Yang, "Dynamic transcriptomic m(6)A decoration: writers, erasers, readers and functions in RNA metabolism," *Cell Research*, vol. 28, no. 6, pp. 616–624, 2018.

[27] G. Jia, Y. Fu, and C. He, "Reversible RNA adenosine methylation in biological regulation," *Trends in Genetics*, vol. 29, no. 2, pp. 108–115, 2013.

[28] K. D. Meyer and S. R. Jaffrey, "The dynamic epitranscriptome: N6-methyladenosine and gene expression control," *Nature Reviews Molecular Cell Biology*, vol. 15, no. 5, pp. 313–326, 2014.

[29] J. A. Bokar, M. E. Shambaugh, D. Polayes, A. G. Matera, and F. M. Rottman, "Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N6-adenosine)-methyltransferase," *RNA*, vol. 3, no. 11, pp. 1233–1247, 1997.

[30] S. Lin, J. Choe, P. Du, R. Triboulet, and R. I. Gregory, "The m(6)A methyltransferase METTL3 promotes translation in human cancer cells," *Molecular Cell*, vol. 62, no. 3, pp. 335–345, 2016.

[31] Y. Du, G. Hou, H. Zhang et al., "SUMOylation of the m6A-RNA methyltransferase METTL3 modulates its function," *Nucleic Acids Research*, vol. 46, no. 10, pp. 5195–5208, 2018.

[32] J. Tong, R. A. Flavell, and H. B. Li, "RNA m(6)A modification and its function in diseases," *Frontiers of Medicine*, vol. 12, no. 4, pp. 481–489, 2018.

[33] X. L. Ping, B. F. Sun, L. Wang et al., "Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase," *Cell Research*, vol. 24, no. 2, pp. 177–189, 2014.

[34] S. Yan, G. Liu, C. Jin et al., "MicroRNA-6869-5p acts as a tumor suppressor via targeting TLR4/NF-kappaB signaling pathway in colorectal cancer," *Journal of Cellular Physiology*, vol. 233, no. 9, pp. 6660–6668, 2018.

[35] B. Hoesel and J. A. Schmid, "The complexity of NF-κB signaling in inflammation and cancer," *Molecular Cancer*, vol. 12, p. 86, 2013.

[36] S. C. Gupta, C. Sundaram, S. Reuter et al., "Inhibiting NF-κB activation by small molecules as a therapeutic strategy," *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, vol. 1799, no. 10-12, pp. 775–787, 2010.

[37] Y. Wang, F. Zheng, G. Gao et al., "MiR-548a-3p regulates inflammatory response via TLR4/NF-κB signaling pathway in rheumatoid arthritis," *Journal of Cellular Biochemistry*, vol. 120, pp. 1133–1140, 2018.

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Research Article

miR-221 Alleviates the Ox-LDL-Induced Macrophage Inflammatory Response via the Inhibition of DNMT3b-Mediated NCoR Promoter Methylation

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Atherosclerosis (AS) is a chronic inflammatory disease, and macrophages play a key role in all phases of AS. Recent studies have shown that miR-221 is a biomarker for AS and stroke; however, the role and mechanism of miR-221 in AS are unclear. Herein, we found that miR-221 and NCoR levels were decreased in ox-LDL-treated THP-1-derived macrophages. In contrast, DNMT3b, IL-6, and TNF-α expression levels were increased under these conditions. Upregulation of miR-221 or NCoR could partially inhibit ox-LDL-induced IL-6 and TNF-α expression. Further studies showed that DNMT3b was a target of miR-221. DNMT3b inhibition also suppressed IL-6 and TNF-α expression and increased NCoR expression in the presence of ox-LDL. Moreover, DNMT3b was involved in ox-LDL-induced DNA methylation in the promoter region of NCoR. These findings suggest that miR-221 suppresses ox-LDL-induced inflammatory responses via suppressing DNMT3b-mediated DNA methylation in the promoter region of NCoR. These results provide a rationale for using intracellular miR-211 as a possible antiatherosclerotic target.

1. Introduction

Atherosclerosis (AS) and its complications, such as myocardial infarction and stroke, are major life-threatening diseases worldwide and impose a heavy financial burden on patients and their families [1]. Macrophages mediating the inflammatory response play pivotal roles throughout the entire process of AS, from initiation to progression, including roles in arterial endothelial damage, atherosclerotic plaque formation, and plaque rupture [2, 3]. Therefore, inhibition of the inflammatory response could delay plaque formation and AS progression [4–8].

MicroRNAs (miRNAs) are short noncoding RNAs that play a major role in controlling the metabolism, function, and fate of eukaryotes via target gene posttranscriptional regulation. The abnormal expression and location of miRNAs at certain times are involved in the occurrence and progression of various diseases, including cancer, neurodevelopmental diseases, autoimmune diseases, and inflammation [9]. Several studies have shown that miRNAs play a pivotal role in the regulation of cholesterol homeostasis, atherosclerosis development, and plaque formation and rupture [10]. Our previous study showed that miR-155 mediated the inflammatory response and plaque formation in an AS mouse model [8]. Recent studies have shown that miR-221 is a biomarker for AS, stroke, local atherosclerotic behavior, and plaque stability [11–13]. Another study demonstrated that miR-221 overexpression blocked lncRNA growth arrest-specific 5 (GAS5), which enhanced the ox-LDL macrophage inflammatory response [14]. However, the role and precise mechanism of miR-221 in the inflammatory response remain unknown.

DNA methylation is a type of epigenetic alteration that occurs in eukaryotes after exposure to various stimuli. This

process involves DNA methyltransferases (DNMTs), such as DNMT3A and DNMT3B, binding to a cytosine nucleotide at a CpG site via a methyl group, forming 5-methylcytosine (5mC) and resulting in gene transcription suppression [15]. Previous studies have shown that abnormal DNA methylation in gene promoter regions is commonly related to AS [16–20]. Bakshi et al. showed that the methylation levels of STAT1, IL12b, MHC2, iNOS, JAK1, and JAK2 were higher in coronary artery disease (CAD) patients than in control subjects [21]. Another study showed increased levels of the DNA demethylase TET1 and decreased levels of DNMT1 in atherosclerotic plaques [22]. Furthermore, inhibition of the promoter methylation of estrogen receptor (ER) α via miR-152 binding to DNMT1 increased the ER expression and had an antiatherosclerotic effect via suppressing human aortic smooth muscle cell (HASMC) proliferation [23]. Therefore, regulating the DNA methylation state in a gene's promoter region is a novel strategy for preventing AS progression.

Nuclear receptor corepressor (NCoR) is a major component of corepressor complexes, which contain histone deacetylase-3 (HDAC), transducin beta-like protein-1 (TBL1), and its receptor TBLR1. This complex plays an important role in nuclear receptor transcription suppression by binding to the promoter region of unliganded nuclear receptors, such as the thyroid hormone receptor (TR) [24–27]. Wagner and colleagues showed that NCoR suppressed human progesterone receptor (PR) transcriptional activity and 8-bromo-cAMP disrupted the interaction between PR and NCoR and enhanced PR transcriptional activity [28]. Similarly, the deletion of USP44, an integral component of NCoR, impaired the ability of NCoR to regulate gene expression and suppressed breast cancer cell invasiveness [29]. Therefore, NCoR plays an important role in regulating gene expression and cellular function.

In this study, we evaluated the expression of miR-221 and the promoter methylation of NCoR after THP-1-derived macrophages were exposed to ox-LDL to identify the novel mechanism by which miR-221 regulates the ox-LDL-induced inflammatory response. Here, we report that ox-LDL suppresses the expression of miR-221 and promotes DNA methylation of the NCoR promoter. miR-221 overexpression suppressed ox-LDL-induced inflammatory responses via binding the target gene DNMT3b and increasing the NCoR expression. Taken together, our data suggest that miR-221 may play a key role in a novel regulatory mechanism that modulates NCoR signaling and the underlying pathology of AS.

2. Methods and Materials

2.1. Materials and Reagents. RPMI-1640 culture medium, DMEM, Opti-MEM™ Reduced Serum Medium (Opti-MEM medium), fetal bovine serum (FBS), and trypsin containing 2.21 mM EDTA were obtained from GIBCO (Shanghai, China). Ox-LDL was obtained from Peking Union-Biology Co. Ltd. (Beijing, China). ViaFect™ Transfection Reagent, miRNA First Strand cDNA Synthesis Kit, Universal Quantitative PCR Master Mix, Luciferase Reporter Gene Assay Kit, DNeasy Blood & Tissue Kit, and bisulfite treatment DNA Methylation™ Kit were purchased from Promega Biotech Co. Ltd. (Beijing, China). T-PER™ Tissue Protein Extraction Reagent was purchased from ThermoFisher Co. Ltd. (Shanghai, China). A Nuclear and Cytoplasmic Protein Extraction Kit was purchased from Beyotime Biotechnology (Nantong, China). Polyvinylidene fluoride (PVDF) membranes and Immobilon Western Chemiluminescence HRP Substrate (ECL kit) were purchased from Merck Millipore Co. Ltd. (Shanghai, China). O-Tetradecanoylphorbol-13-acetate (PMA), TRIzol reagent, and other reagents were purchased and used as received from Sigma-Aldrich (Shanghai, China).

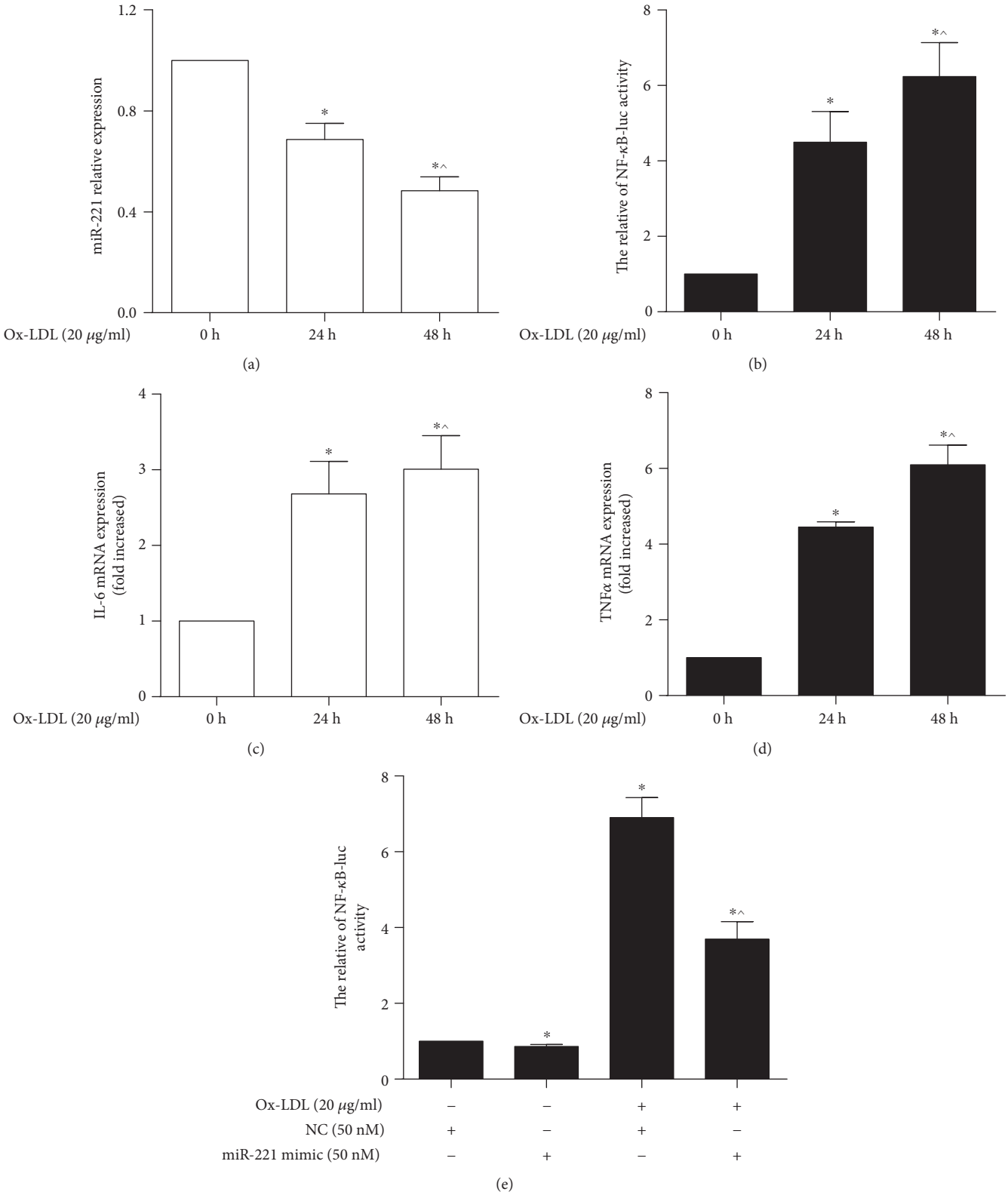
2.2. Cell Culture, Differentiation, and Ox-LDL Treatment. THP-1 cells and 293T cells were kindly provided by the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China). THP-1 cells were cultured in a RPMI-1640 medium with 10% FBS and 1% antibiotics. THP-1 cells were treated with 100 nM PMA for 48 h to induce macrophage differentiation [30, 31]. Macrophages were treated with 20 μg/ml ox-LDL for the indicated times.

293T cells were cultured in DMEM with 10% FBS. Cells were passaged by trypsinization with 0.25% trypsin and seeded onto cell culture plates for further study.

2.3. Transient Transfection with miR-221 Mimic and Inhibitor. miR-221 mimic and inhibitor sequences were 5′-ACCUGGCAUACAAUGUAGAUUU-3′ and 5′-AGCT AAAAAAGCTACATT GTCTGCTGGGTTTCG-3′, respectively. The negative control (NC) sequence was 5′-UUCUCC GAACGUGUCACGUTT-3′. All oligos were synthesized by GenePharma (Shanghai, China). THP-1 cells were seeded into 6-well plates and cultured overnight. These cells were transfected with 100 nM miR-221 mimic or inhibitor and 50 nM NC using ViaFect™ Transfection Reagent for 48 h. Then, these cells were differentiated into macrophages for further study.

2.4. DNMT3b Silencing and NCoR Overexpression. The DNMT3b siRNA sequences were 5′-CACTGGTTCTGCGC TGGGA-3′ (siRNA-1), 5′-GGGUUAAAGCGGAGAC UCUTT-3′ (siRNA-2), and 5′-GCUGUCCGAACUCGAA AUATT-3′ (siRNA-3). These siRNAs and the NC were transfected into THP-1 cells for 48 h according to the manufacturer's instructions and differentiated into macrophages for further study.

An NCoR overexpression adenovirus (adv. NCoR) and empty vector adenovirus (MOCK) were produced and purified according to standard techniques by Hanbio Inc. (Shanghai, China). THP-1 cells were seeded into 6 cm dishes and cultured for 24 h. Then, adv. NCoR (multiplicity of infection (MOI) = 200) and MOCK (MOI = 100) were used to infect the THP-1 cells for 24 h. The culture medium was discarded, and a fresh medium was added to culture for an additional 24 h. These cells were differentiated into macrophages with PMA.



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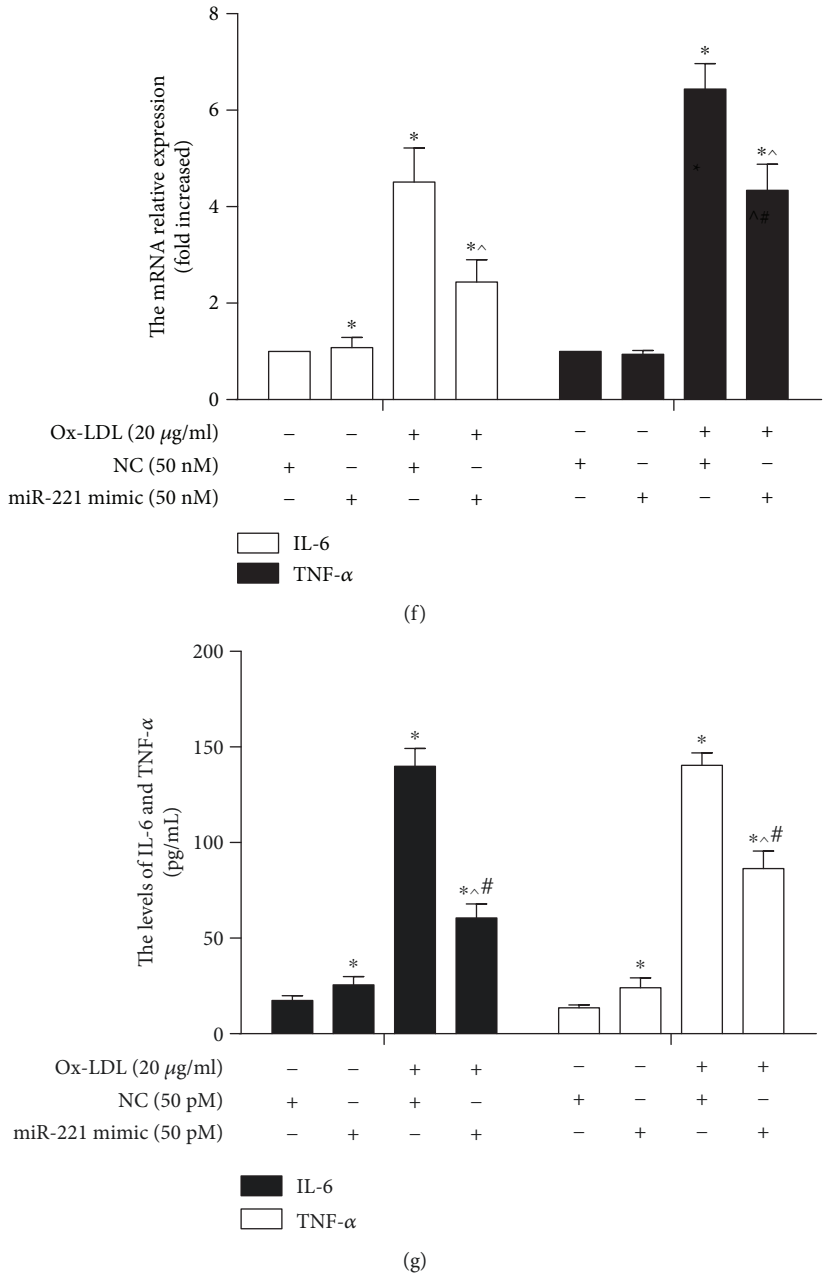


Figure 1: miR-221 suppressed the ox-LDL-induced macrophage inflammatory response. (a–d) The expression of miR-221, IL-6, and TNFα and activity of NF-κB were detected by qPCR after THP-1-derived macrophages were treated with ox-LDL at the indicated times ($n = 3$). $^{*}p < 0.05$, vs. the 0 h group; $^{\wedge}p < 0.05$, vs. the 24 h group. (e–g) NF-κB activity and IL-6 and TNF-α expression were evaluated by a luciferase assay, qPCR, and flow cytometry (FCM) after THP-1 cells were transfected with miR-221 mimic for 48 h, differentiated into macrophages, and incubated with ox-LDL for another 24 h ($n = 3$). $^{*}p < 0.05$, vs. the NC group; $^{\wedge}p < 0.05$, vs. the ox-LDL/NC group; $^{\#}p < 0.05$, vs. the miR-221 mimic group.

2.5. Total RNA Isolation and Real-Time PCR. The treated cells were harvested, and total RNA was isolated using TRIzol reagent according to the manufacturer's protocol. A miRNA First Strand cDNA Synthesis Kit and Universal Quantitative PCR (qPCR) Master Mix were used to evaluate the expression of miR-221 according to the manufacturer's protocol. U6 was used as a control for miR-221 normalization. For mRNA analysis, cDNA was generated using a RevertAid First Strand cDNA Synthesis Kit and qPCR was conducted using the BIO-RAD CFX96 system. GAPDH was used as an internal control. All data were analyzed using the $2^{-\Delta\Delta t}$ method. The primers used for these assays are shown in Supplementary Table 1.

2.6. Western Blotting. Total and nuclear protein from treated cells were collected using T-PER™ Tissue Protein Extraction Reagent and a Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's protocol. The protein extracts were separated by SDS-PAGE and then electrophoretically transferred onto PVDF membranes. The PVDF

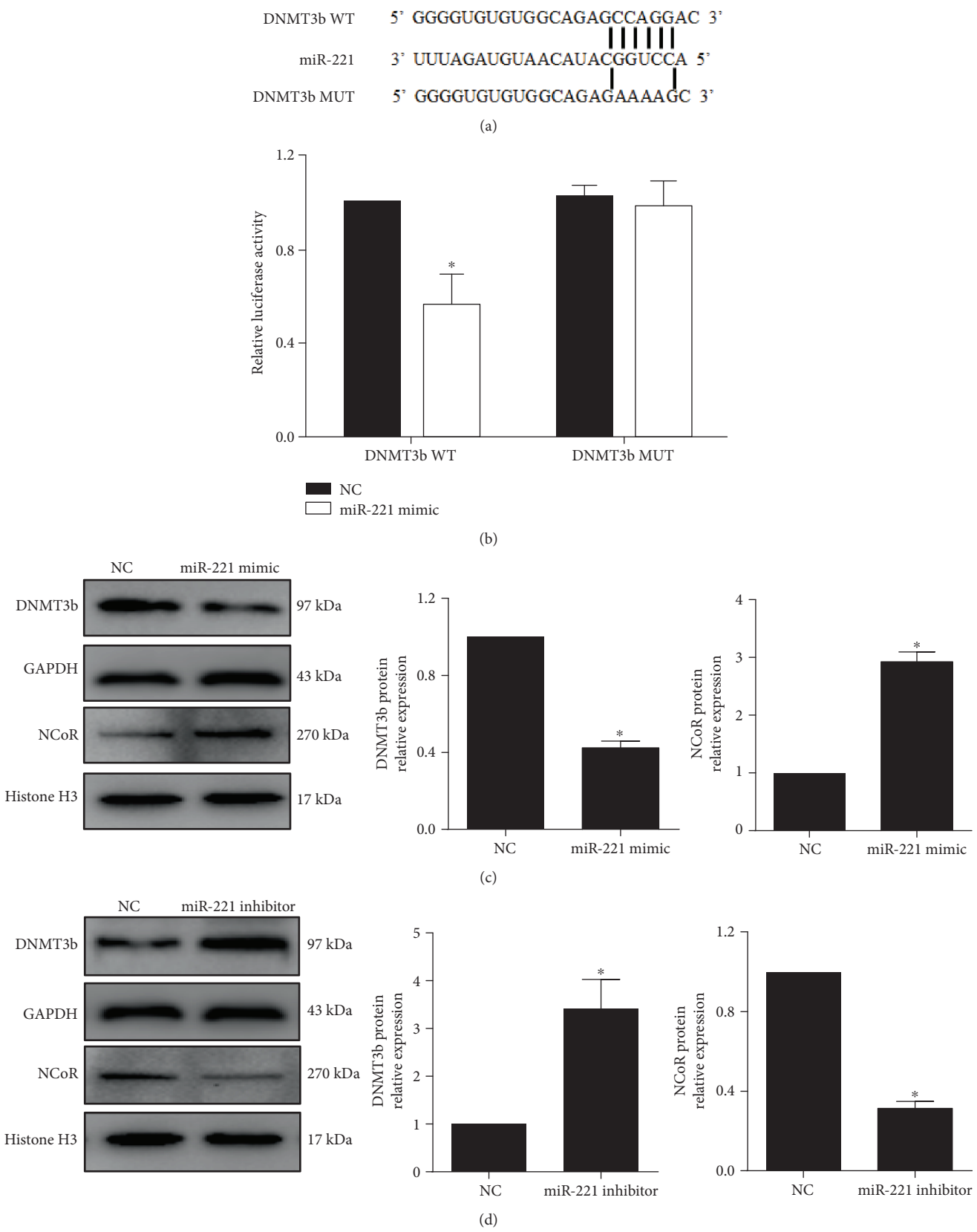
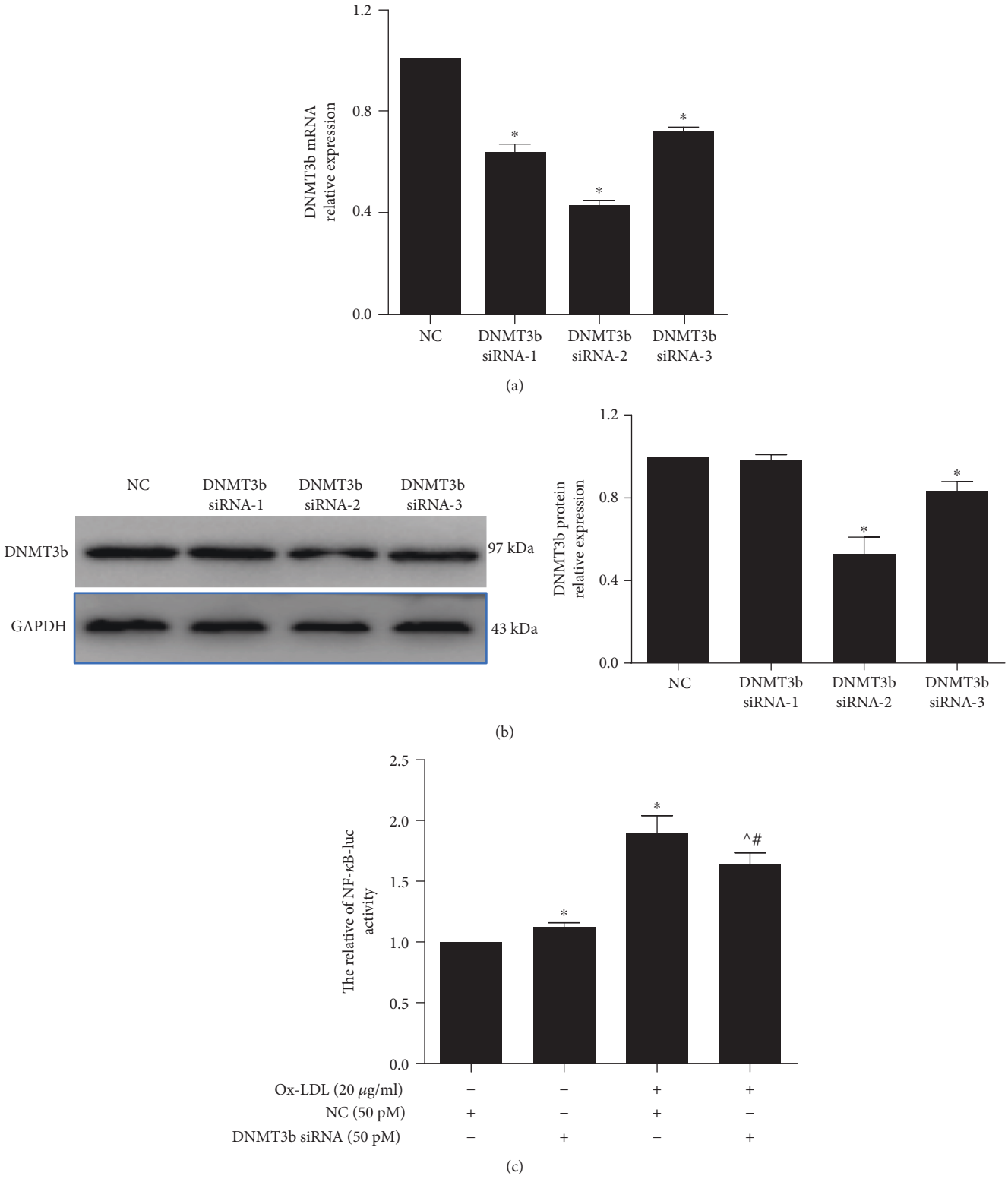


Figure 2: DNMT3b is a direct target of miR-221. (a) Predicted alignment between the miR-221 sequence and the wild-type (WT) and mutated (MUT) 3′UTRs of DNMT3b. (b) A luciferase assay evaluated the reporter activity of the DNMT3b WT and MUT 3′UTRs in 293T cells ($n = 3$). $^*p < 0.05$, vs. the NC group. (c, d) Western blot detected the protein expression of DNMT3b and NCoR after THP-1-derived macrophages were transfected with miR-221 mimic or inhibitor for 48 h ($n = 3$). $p < 0.05$, vs. the NC group.



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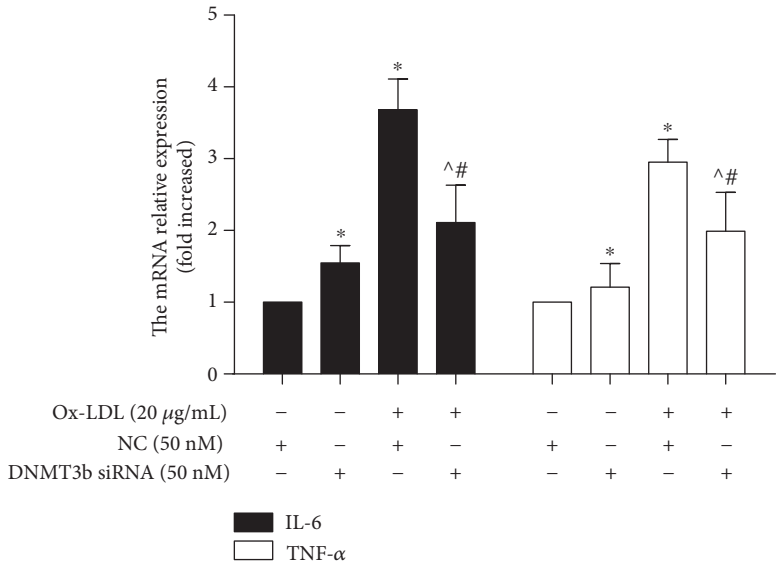

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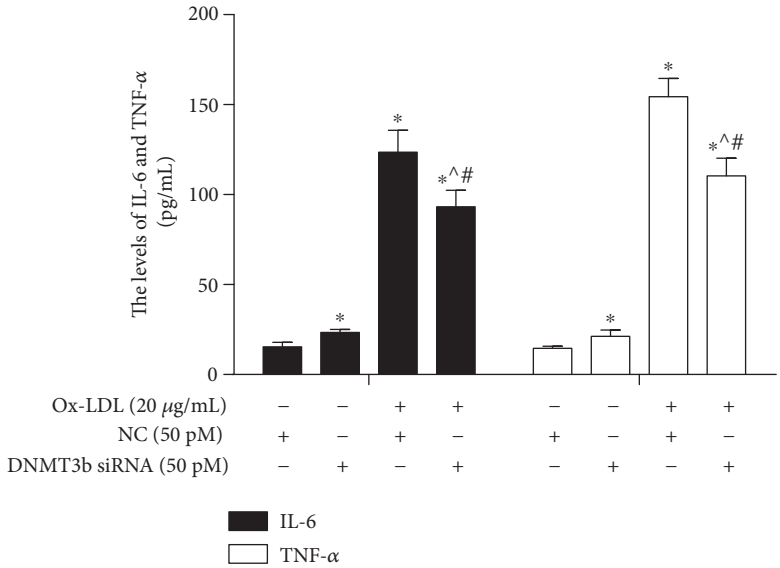

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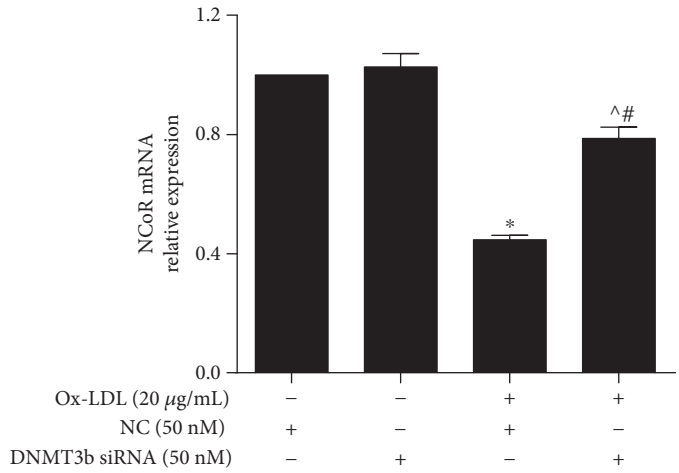

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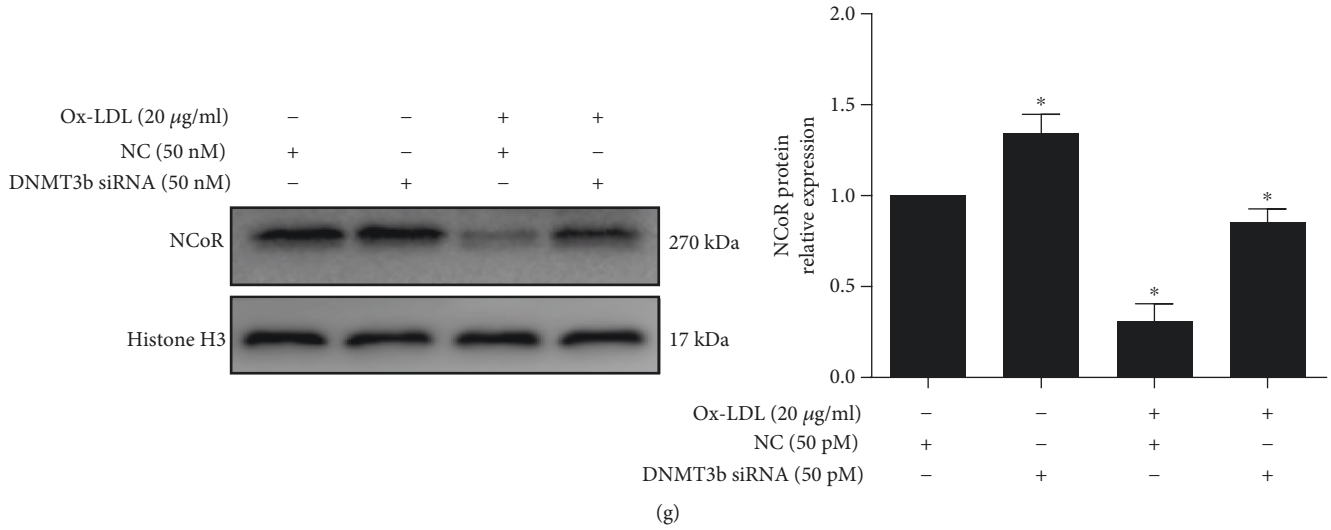


Figure 3: Silencing DNMT3b attenuated ox-LDL-induced macrophage inflammatory responses via increased NCoR. (a, b) DNMT3b mRNA and protein expression was evaluated by qPCR and western blotting after THP-1 cells were transfected with DNMT3b siRNAs for 48 h and induced with PMA for 48 h ($n = 3$). $^{*}p < 0.05$, vs. the NC group. THP-1 cells were transfected with DNMT3b siRNA and NC for 48 h and pretreated with PMA for 48 h. These cells were treated with ox-LDL for 24 h. NF-κB activity (c), IL-6 and TNF-α mRNA expression (d), IL-6 and TNF-α levels in culture supernatant (e), NCoR mRNA levels (f), and protein levels (g) were analyzed using a luciferase assay, qPCR, FCM, and western blotting, respectively ($n = 3$). $^{*}p < 0.05$, vs. the NC group; $^{\wedge}p < 0.05$, vs. the ox-LDL/NC h group; $^{\#}p < 0.05$, vs. the DNMT3b siRNA group.

membranes were incubated with primary antibodies against DNMT3B (CST, lot: #57868) (1 : 500), NCoR (CST, lot: #34271) (1 : 1000), GAPDH (CST, lot: #5174) (1 : 2000), and histone H3 (CST, lot: #4499) (1 : 1000) overnight at 4°C. HRP-labeled secondary antibodies were incubated with the membranes and detected using an ECL system.

2.7. NF-κB Activity Assay. The luciferase reporter pNF-κB was transfected into THP-1 cells using ViaFect™ Transfection Reagent for 48 h, and these cells were then incubated in a culture medium with G418. The selective medium was changed every 2 d until resistant clones appeared. The selected clones (luc-pNF-κB-THP1) were maintained in a fresh G418-containing medium for analysis and further experiments. pRL Renilla Luciferase control reporter vectors were transfected into luc-pNF-κB-THP1 cells, and luciferase assays were carried out according to the manufacturer's protocol.

2.8. Methylation-Specific PCR (MSP). The CpG island in the promoter region of NCoR was analyzed, and an MSP primer was designed with the MethPrimer 2.0 website [32]. Genomic cDNA from macrophages was prepared with a DNeasy Blood & Tissue Kit and treated with bisulfite using an EZ DNA Methylation™ Kit. Then, the bisulfite samples were amplified by PCR. The primers used for this assay are shown in Supplementary Table 1.

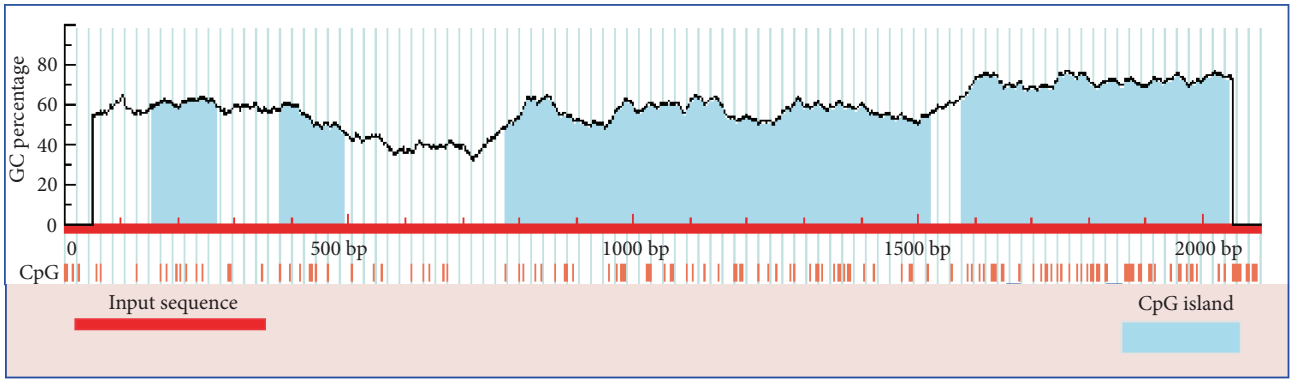
2.9. Luciferase Reporter Assay. Wild-type and mutation sequences in the 3′UTR of DNMT3b were synthesized and inserted into the SpeI and HindIII sites of the pMIR-reporter luciferase vector. The two plasmid constructs were validated by sequencing. The details of the luciferase reporter assay have been described in our previous study [8].

2.10. Quantification of IL-6 and TNF-α in Macrophage Culture Supernatants. IL-6 and TNF-α levels in macrophage culture supernatants were quantified with the BioLegend LEGENDplex™ Kit according to the manufacturer's instructions [33]. Briefly, culture supernatants were collected after macrophages were treated as indicated. The supernatants were incubated with LEGENDplex beads for 2 h and then with antibodies and streptavidin-PE. The beads were analyzed by flow cytometry, and the data were analyzed using LEGENDplex software (BioLegend).

2.11. Statistical Analysis. The data are expressed as the mean ± standard error (S.E.) and were from at least three independent experiments. Two-tailed Student's t-test and one-way analysis of variance (ANOVA) were performed. Significant differences were defined as $p < 0.05$.

3. Results

3.1. miR-221 Suppressed the Ox-LDL-Induced Inflammatory Response in Macrophages. In this study, we investigated miR-221 expression after THP-1 cell-differentiated macrophages were treated with ox-LDL (Figure 1(a)). The data showed that miR-221 expression was lower in the 24 h and 48 h groups than in the 0 h group ($p < 0.05$), and ox-LDL induced miR-221 in a dose-dependent manner. Consistent with the findings of a previous study, NF-κB activity and IL-6 and TNF-α mRNA levels were increased after cells were treated with ox-LDL (Figures 1(b)–1(d)). Moreover, NF-κB activity was lower in the ox-LDL/miR-221 mimic group than



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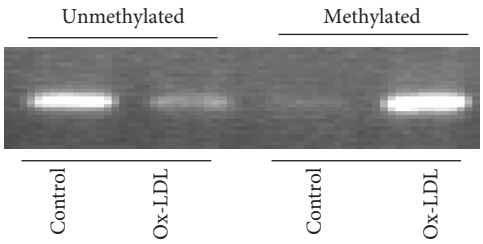

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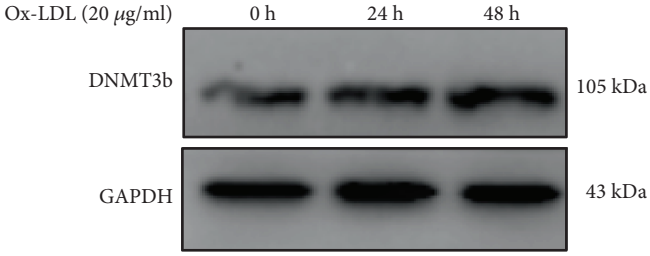

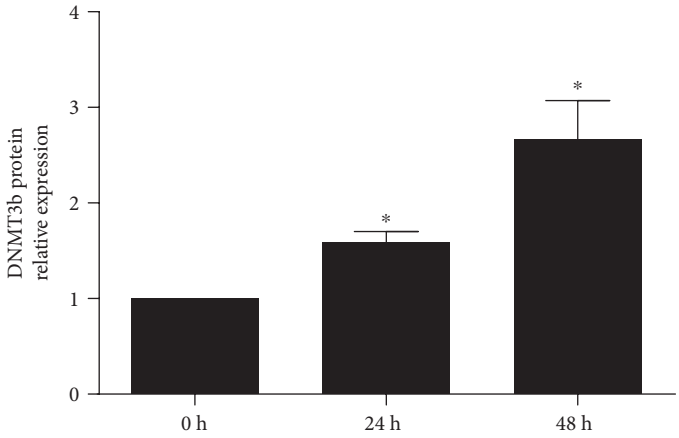

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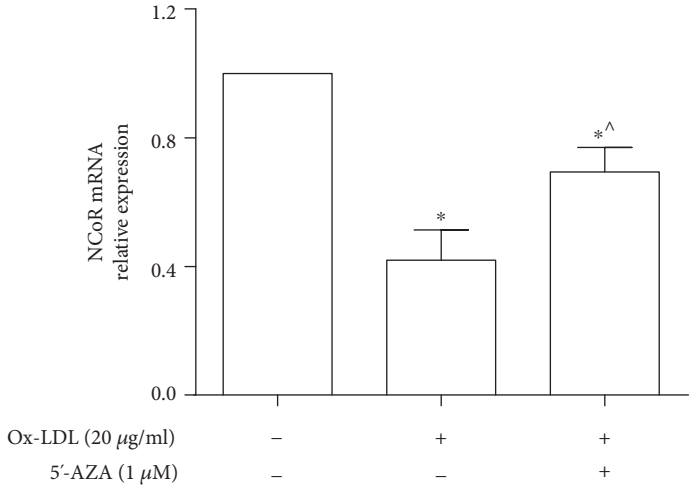

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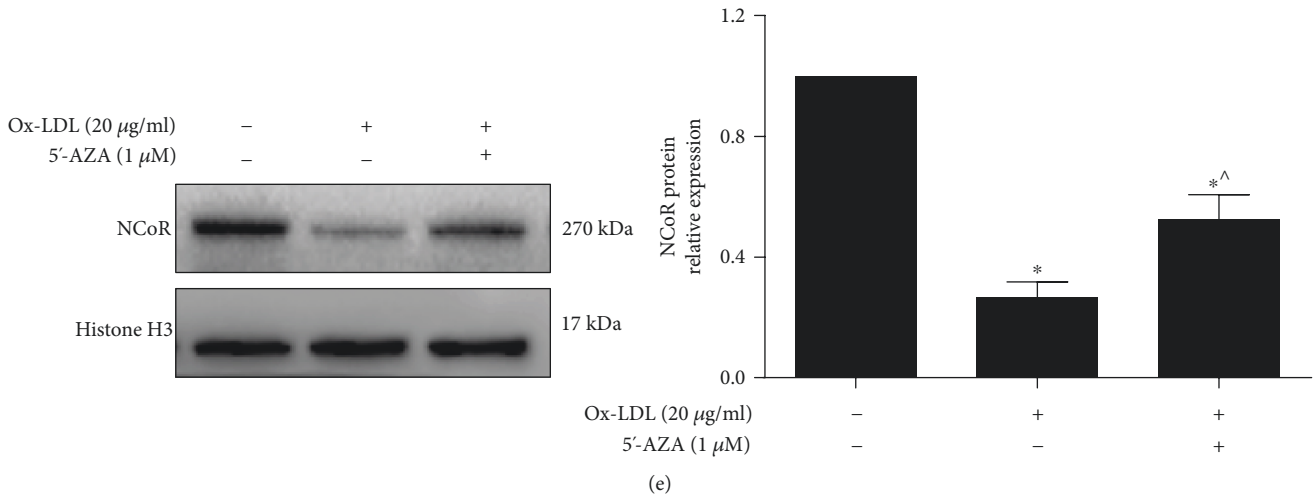


Figure 4: DNMT3b is involved in the ox-LDL-mediated promoter methylation of NCoR. (a) Schematic of the CpG island in the promoter region of NCoR. (b) MSP was used to analyze DNA methylation in the NCoR promoter region ($n = 3$). (c) Western blot analysis of the protein expression of DNMT3b after THP-1-derived macrophages were treated with ox-LDL ($n = 3$). $^*p < 0.05$, vs. the NC group; (d, e) the protein and mRNA expression of NCoR was evaluated by qPCR and western blotting after THP-1-derived macrophages were stimulated with ox-LDL in the presence or absence of 5′-AZA ($n = 3$). $^*p < 0.05$, vs. the NC group; $^\wedge p < 0.05$, vs. the ox-LDL group.

in the ox-LDL/NC group ($p < 0.05$). There was no difference between the NC group and the miR-221 mimic alone group ($p > 0.05$) (Figure 1(e)). Inflammatory mediators were detected after the macrophages were treated with miR-221 mimic and ox-LDL. miR-221 upregulation could partly reverse the increases in IL-6 and TNF-α expression induced by 20 μg/ml ox-LDL and NC (Figures 1(f) and 1(g)) ($p < 0.05$). These data suggested that miR-221 suppressed the production of inflammatory mediators.

3.2. DNMT3b Is a Target Gene of miR-221. miR-221 regulates the inflammatory response via a direct target. In this study, luciferase plasmids containing wild-type (WT) and mutated (Mut) DNMT3b 3′UTRs (schematic shown in Figure 2(a)) were transfected into HEK-293T cells, and miR-221 mimic or miR-NC was then transfected into these cells for 24 h. The level of luciferase activity was lower in the DNMT3b WT/miR-221 mimic group than in the DNMT3b WT/NC group ($p < 0.05$). There was no difference in luciferase activity between the DNMT3b MUT/miR-221 mimic and DNMT3b MUT/NC groups ($p > 0.05$) (Figure 2(b)). To further confirm that DNMT3b was a direct target of miR-221, we evaluated the protein expression of DNMT3b and NCoR after macrophages were treated with miR-221 mimic or inhibitor. These data showed decreased DNMT3b and increased NCoR protein levels after the cells were treated with miR-221 mimic (Figure 2(c)). In contrast, DNMT3b protein expression increased, and NCoR protein expression decreased when the cells were treated with miR-221 inhibitor (Figure 2(d)). These data indicate that DNMT3b is a target gene of miR-221.

3.3. DNMT3b Knockdown Partly Reversed Inflammatory Signal Activation in Macrophages. Three DNMT3b siRNA oligos were transfected into THP-1 cells to evaluate the effective suppression of DNMT3b expression, and qPCR and western blotting showed that DNMT3b mRNA and protein levels were significantly decreased. Furthermore, DNMT3b siRNA oligo (02) was more effective in knocking down DNMT3b than the other siRNA oligos (Figures 3(a) and 3(b)). The level of NF-κB activity was lower in the DNMT3b/ox-LDL group than in the NC/ox-LDL group ($p < 0.05$) (Figure 3(c)). The levels of IL-6 and TNF-α were also lower in the DNMT3b/ox-LDL group than in the NC/ox-LDL group ($p < 0.05$) (Figures 3(d) and 3(e)). Furthermore, silencing DNMT3b partly blocked ox-LDL-induced NCoR mRNA and protein expression (Figures 3(f) and 3(g)). Additionally, silencing DNMT3b increased miR-221 expression after the cells were treated with ox-LDL (Supplementary Figure 1). These data suggest that DNMT3b regulates the ox-LDL-mediated macrophage inflammatory response via NCoR.

3.4. Ox-LDL Promoted DNA Methylation of the NCoR Promoter by DNMT3b. DNA methylation in promoter regions is an important mechanism for regulating gene expression. Herein, a CpG island in the promoter of NCoR was predicted by the Li Lab website (http://www.urogene.org/index.html) [30], and the scheme is shown in Figure 4(a). An MSP assay showed that ox-LDL induced DNA methylation of the NCoR promoter (Figure 4(b)). Additionally, the protein expression of DNMT3b was notably increased after macrophages were treated with ox-LDL (Figure 4(c)). Macrophages were also treated with ox-LDL with or without the demethylating agent 5-Aza-dC. The mRNA and protein expression of NCoR was higher in macrophages in the ox-LDL/5-Aza-dC group than in the ox-LDL alone group ($p < 0.05$) (Figures 3(d) and 3(e)). These data suggest that ox-LDL induced DNA methylation of the NCoR promoter with the involvement of DNMT3b.

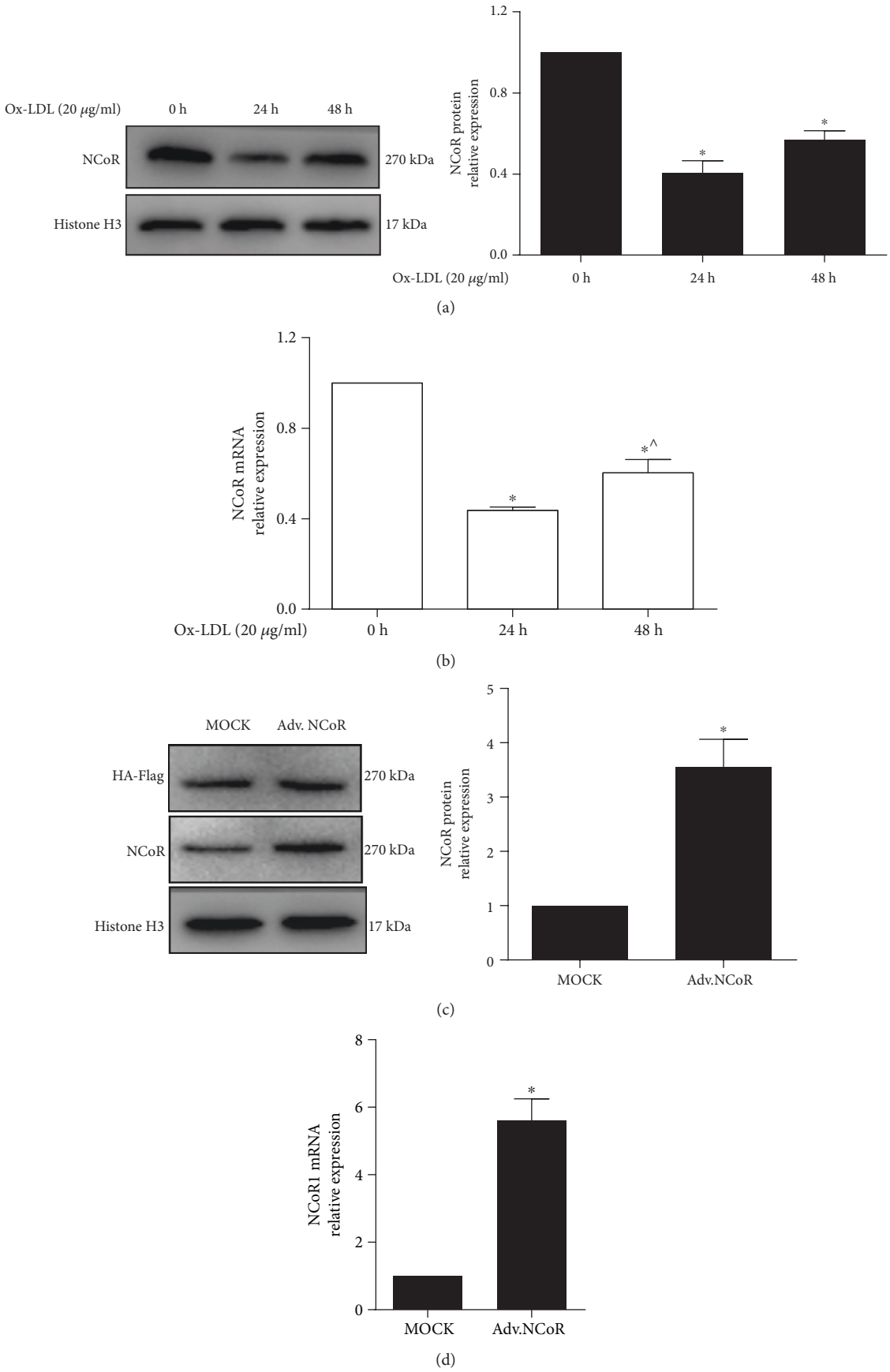
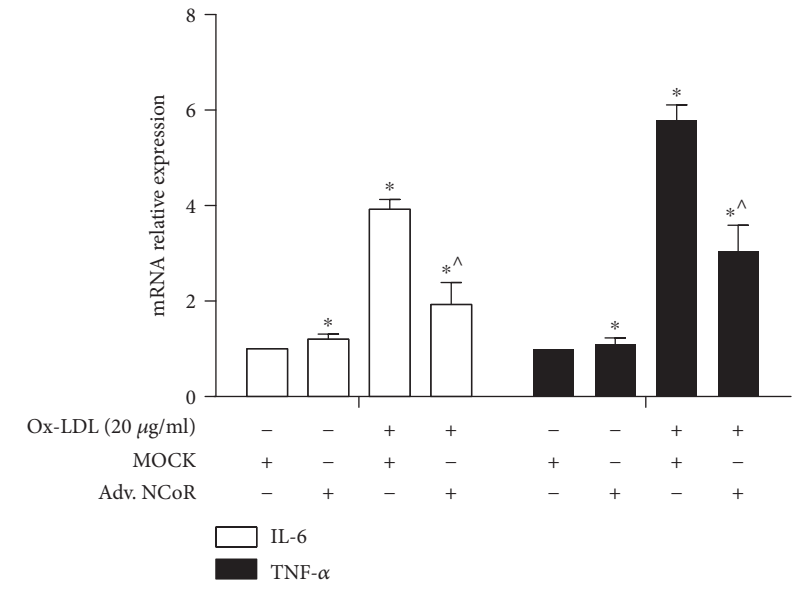
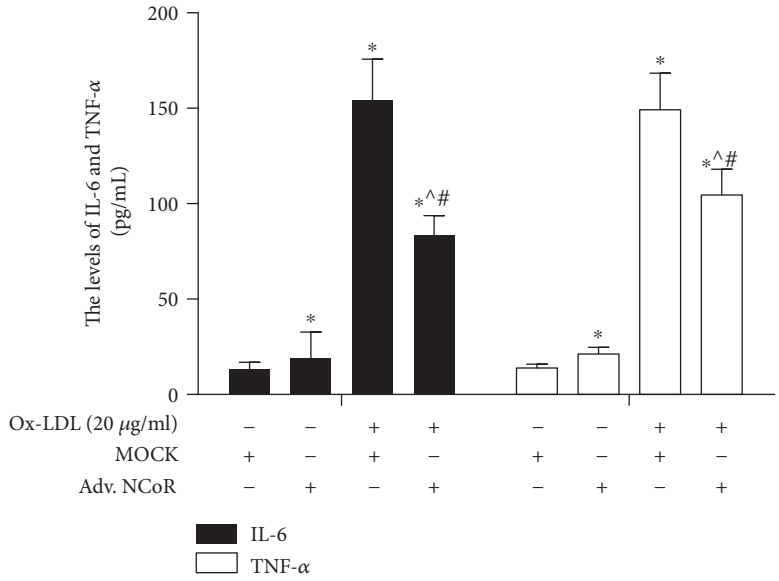


Figure 5: Continued.



(e)



(f)

Figure 5: NCoR overexpression inhibited the ox-LDL-induced inflammatory response in macrophages. (a, b) The protein and mRNA expression levels of NCoR were detected by western blotting and qPCR after THP-1-derived macrophages were treated with ox-LDL at the indicated time points ($n = 3$). $^{*}p < 0.05$, vs. the 0 h group; $^{\wedge}p < 0.05$, vs. the 24 h group. THP-1 cells were transfected with adv. HA-NCoR for 48 h and treated with PMA for 48 h. (c, d) The protein and mRNA expression levels of NCoR were evaluated after THP-1 cells were transfected with adv. NCoR for 48 h ($n = 3$). $^{*}p < 0.05$, vs. the MOCK group. (e, f) The levels of IL-6 and TNF-α in culture supernatants and mRNA were measured by qPCR and FCM after macrophages were treated with ox-LDL for 24 h ($n = 3$). $^{*}p < 0.05$, vs. the NC group; $^{\wedge}p < 0.05$, vs. the ox-LDL/NC group.

3.5. NCoR Alleviated the Production of IL-6 and TNF-α. In this study, the mRNA and protein expression levels of NCoR were evaluated after macrophages were treated with 20 μg/ml ox-LDL. The data showed that the mRNA and protein expression levels of NCoR in this group were lower than in the 0 h group ($p < 0.05$) (Figures 5(a) and 5(b)). To explore the role of NCoR in the ox-LDL-induced inflammatory response, macrophages were infected with adv. HA-NCoR or adv. HA (MOCK) for 48 h. Figures 5(c) and 5(d) show that the protein and mRNA expression levels of NCoR were obviously higher in the adv. NCoR group than in the MOCK group ($p < 0.05$). Furthermore, the expression levels of IL-6 and TNF-α were lower in the ox-LDL/adv. NCoR group than in the ox-LDL/MOCK group ($p < 0.05$) (Figures 5(e) and 5(f)); moreover, the expression of miR-221 was not significantly changed (Supplementary Figure 2). These data

show that NCoR alleviated the production of IL-6 and TNF-α, suggesting that NCoR suppressed the ox-LDL-induced inflammatory response.

4. Discussion

In this study, we reported that miR-221 upregulation could partially inhibit the ox-LDL-induced inflammatory response. We have shown that miR-221 regulates NCoR expression by directly binding to DNMT3b and suppressing its DNA methylation activity, resulting in the suppression of inflammatory mediator production induced by ox-LDL in macrophages.

The role of miR-221 in immune and inflammatory responses is controversial. Zhao et al. demonstrated that lipopolysaccharide (LPS) induced miR-221 expression, and miR-221 overexpression strengthened LPS-induced NF-κB activation and increased TNF-α and IL-6 levels via binding the target gene A20 [34]. In endothelial cells, miR-221 upregulation promoted the inflammatory response in an NF-κB-dependent manner [35–37]. In contrast, miR-221 overexpression plays an anti-inflammatory role in endothelial cells via reducing p38/NF-κB levels [38]. miR-221 also binds the TNF-α $3'$UTR and promotes its degradation [39]. miR-221 overexpression blocked lncRNA GAS5, which enhanced the ox-LDL macrophage inflammatory response [14]. Another study showed that miR-221 overexpression suppressed the ox-LDL macrophage inflammatory response [14]. In this study, we identified that miR-221 plays a key role in regulating the macrophage inflammatory response. We found that ox-LDL suppressed miR-221 expression and increased NF-κB promoter activity and IL-6 and TNF-α levels. Consistent with the results of other studies [14], miR-221 overexpression partially suppressed the ox-LDL-induced activation of NF-κB and inflammatory mediator production. Furthermore, miR-221 upregulation also increased NCoR levels. These data suggest that miR-221 plays an anti-inflammatory role in ox-LDL-induced macrophage inflammatory responses by increasing NCoR levels and suppressing the activity of the NF-κB promoter.

DNA methyltransferases mediate the covalent addition of a methyl group to cytosine residues within CpG dinucleotides, resulting in DNA methylation in the promoter region of a gene. Yu et al. found that ox-LDL induced DNMT1 in macrophages and that increased DNMT1 levels promoted AS progression via hypermethylation of the peroxisome proliferation-activated receptor (PPAR) promoter [40]. Herein, we found that ox-LDL induced DNMT3b in macrophages, and silencing DNMT3b increased NCoR expression, thus inhibiting NF-κB promoter activation and decreasing inflammatory mediator levels. Furthermore, DNMT3b was a target of miR-221. Given the abovementioned data, these findings suggest that miR-221 inhibited the ox-LDL inflammatory response by suppressing DNMT3b-mediated hypermethylation of the NCoR promoter.

Several studies have reported that NCoR plays an important role in regulating the inflammatory response. NCoR is located in the promoter regions of inflammatory pathway genes, such as NF-κB and AP-1, and maintains a suppressive state in the absence of ligands [41, 42]. After inflammatory pathway activation by TLR4 or TLR2, NCoR is detached from the promoter region of proinflammatory transcription factors, resulting in increased gene expression and inflammatory mediator production [43]. Furthermore, the regulation of NCoR expression also controls the inflammatory response [44, 45]. In the present study, we found that NCoR levels were decreased upon exposure to ox-LDL and that the NCoR promoter was hypermethylated. Moreover, 5-AZA, an inhibitor of DNA methyltransferase, restored NCoR expression after cells were treated with ox-LDL, suggesting that the downregulation of NCoR may inhibit the transcriptional activity of the NCoR promoter. Barish et al. showed that Bcl6-SMRT/NCoR complex suppressed the transcriptional activation of NF-κB, constrained ox-LDL-induced macrophage inflammatory responses, and prevented AS progression [46]. Additionally, minimally oxidized LDL-induced NCoR removal from chemokine promoters promotes the transcription of inflammatory cytokines within atherosclerotic lesions [47]. Consistent with the findings of previous studies, NCoR overexpression in THP-1-derived macrophages also partly reversed the ox-LDL-mediated induction of IL-6 and TNF-α expression. These data suggest that restoring NCoR levels is an effective approach against the inflammatory response.

In conclusion, miR-221 suppressed the inflammatory response by downregulating DNMT3b-mediated DNA methylation in the promoter region of NCoR and played a critical role in atherogenesis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Lixia Yang and Jinshan Ye designed the study and the experiments. Jinshan Ye, Ruiwei Guo, Wenjun Zeng, and Yanan Duan were responsible for data collection. Jinshan Ye and Zhihua Yang analyzed the data. Jinshan Ye drafted the manuscript. Jinshan Ye and Lixia Yang revised the manuscript, and all authors approved the final manuscript. Jinshan Ye and Yaxi Wu contributed equally to this work.

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Supplementary Materials

Supplementary Table 1: the sequence of PCR primer. Supplementary Figure 1: qPCR analyzed the expression of miR-221. THP-1 cells were transfected with DNMT3b siRNA and NC

for 48 h and pretreated with PMA for 48 h. These cells were treated with ox-LDL for 24 h. $^{*}p < 0.05$, vs. the NC group; $^{\wedge}p < 0.05$, vs. the ox-LDL/NC h group. Supplementary Figure 2: qPCR analyzed the expression of miR-221. THP-1 cells were transfected with adv. HA-NCoR for 48 h and treated with PMA for 48 h. These cells were treated with ox-LDL for 24 h. $^{*}p < 0.05$, vs. the MOCK group; $^{\wedge}p < 0.05$, vs. the ox-LDL/NC h group. *(Supplementary Materials)*

References

[1] W. Martinet, I. Coornaert, P. Puylaert, and G. R. Y. de Meyer, "Macrophage death as a pharmacological target in atherosclerosis," *Frontiers in Pharmacology*, vol. 10, p. 306, 2019.

[2] P. Libby, Y. Okamoto, V. Z. Rocha, and E. Folco, "Inflammation in atherosclerosis: transition from theory to practice," *Circulation Journal*, vol. 74, no. 2, pp. 213–220, 2010.

[3] V. Sarrazy, S. Sore, M. Viaud et al., "Maintenance of macrophage redox status by ChREBP limits inflammation and apoptosis and protects against advanced atherosclerotic lesion formation," *Cell Reports*, vol. 13, no. 1, pp. 132–144, 2015.

[4] A. G. Zaman, G. Helft, S. G. Worthley, and J. J. Badimon, "The role of plaque rupture and thrombosis in coronary artery disease," *Atherosclerosis*, vol. 149, no. 2, pp. 251–266, 2000.

[5] J. Ben, B. Jiang, D. Wang et al., "Major vault protein suppresses obesity and atherosclerosis through inhibiting IKK-NF-κB signaling mediated inflammation," *Nature Communications*, vol. 10, no. 1, p. 1801, 2019.

[6] W. Chen, X. Li, S. Guo et al., "Tanshinone IIA harmonizes the crosstalk of autophagy and polarization in macrophages via miR-375/KLF4 pathway to attenuate atherosclerosis," *International Immunopharmacology*, vol. 70, pp. 486–497, 2019.

[7] A. A. Momtazi-Borojeni, E. Abdollahi, B. Nikfar, S. Chaichian, and M. Ekhlasi-Hundrieser, "Curcumin as a potential modulator of M1 and M2 macrophages: new insights in atherosclerosis therapy," *Heart Failure Reviews*, vol. 24, no. 3, pp. 399–409, 2019.

[8] J. Ye, R. Guo, Y. Shi, F. Qi, C. Guo, and L. Yang, "miR-155 regulated inflammation response by the SOCS1-STAT3-PDCD4 axis in atherogenesis," *Mediators of Inflammation*, vol. 2016, Article ID 8060182, 14 pages, 2016.

[9] A. M. Ardekani and M. M. Naeini, "The role of microRNAs in human diseases," *AvicennaJournal of Medical Biotechnology*, vol. 2, pp. 161–179, 2010.

[10] B. Laffont and K. J. Rayner, "MicroRNAs in the pathobiology and therapy of atherosclerosis," *The Canadian Journal of Cardiology*, vol. 33, no. 3, pp. 313–324, 2017.

[11] P. C. Tsai, Y. C. Liao, Y. S. Wang, H. F. Lin, R. T. Lin, and S. H. H. Juo, "Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease," *Journal of Vascular Research*, vol. 50, no. 4, pp. 346–354, 2013.

[12] A. E. Bildirici, S. Arslan, N. Özbilüm Şahin, Ö. Berkan, O. Beton, and M. B. Yilmaz, "MicroRNA-221/222 expression in atherosclerotic coronary artery plaque versus internal mammarian artery and in peripheral blood samples," *Biomarkers*, vol. 23, no. 7, pp. 670–675, 2018.

[13] H. A. Bazan, S. A. Hatfield, C. B. O'Malley, A. J. Brooks, D. Lightell Jr., and T. C. Woods, "Acute loss of miR-221 and miR-222 in the atherosclerotic plaque shoulder accompanies plaque rupture," *Stroke*, vol. 46, no. 11, pp. 3285–3287, 2015.

[14] J. Ye, C. Wang, D. Wang, and H. Yuan, "LncRBA GSA5, upregulated by ox-LDL, aggravates inflammatory response and MMP expression in THP-1 macrophages by acting like a sponge for miR-221," *Experimental Cell Research*, vol. 369, no. 2, pp. 348–355, 2018.

[15] M. Okano, D. W. Bell, D. A. Haber, and E. Li, "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development," *Cell*, vol. 99, no. 3, pp. 247–257, 1999.

[16] M. O. Laukkanen, S. Mannermaa, M. O. Hiltunen et al., "Local hypomethylation in atherosclerosis found in rabbit *ec-sod* gene," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 9, pp. 2171–2178, 1999.

[17] V. Bollati, J. Schwartz, R. Wright et al., "Decline in genomic DNA methylation through aging in a cohort of elderly subjects," *Mechanisms of Ageing and Development*, vol. 130, no. 4, pp. 234–239, 2009.

[18] A. Baccarelli, R. Wright, V. Bollati et al., "Ischemic heart disease and stroke in relation to blood DNA methylation," *Epidemiology*, vol. 21, no. 6, pp. 819–828, 2010.

[19] S. A. Castillo-Díaz, M. E. Garay-Sevilla, M. A. Hernández-González, M. O. Solís-Martínez, and S. Zaina, "Extensive demethylation of normally hypermethylated CpG islands occurs in human atherosclerotic arteries," *International Journal of Molecular Medicine*, vol. 26, no. 5, pp. 691–700, 2010.

[20] M. O. Hiltunen, M. P. Turunen, T. P. Häkkinen et al., "DNA hypomethylation and methyltransferase expression in atherosclerotic lesions," *Vascular Medicine*, vol. 7, no. 1, pp. 5–11, 2002.

[21] C. Bakshi, R. Vijayvergiya, and V. Dhawan, "Aberrant DNA methylation of M1-macrophage genes in coronary artery disease," *Scientific Reports*, vol. 9, no. 1, p. 1429, 2019.

[22] A. Greißel, M. Culmes, R. Napieralski et al., "Alternation of histone and DNA methylation in human atherosclerotic carotid plaques," *Thrombosis and Haemostasis*, vol. 114, no. 8, pp. 390–402, 2015.

[23] Y. S. Wang, W. W. Chou, K. C. Chen, H. Y. Cheng, R. T. Lin, and S. H. H. Juo, "MicroRNA-152 mediates DNMT1-regulated DNA methylation in the estrogen receptor α gene," *PLoS One*, vol. 7, no. 1, article e30635, 2012.

[24] J. D. Chen and R. M. Evans, "A transcriptional co-repressor that interacts with nuclear hormone receptors," *Nature*, vol. 377, no. 6548, pp. 454–457, 1995.

[25] C. K. Glass and M. G. Rosenfeld, "The coregulator exchange in transcriptional functions of nuclear receptors," *Genes & Development*, vol. 14, no. 2, pp. 121–141, 2000.

[26] J. Zhang, M. Kalkum, B. T. Chait, and R. G. Roeder, "The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2," *Molecular Cell*, vol. 9, no. 3, pp. 611–623, 2002.

[27] H.-. G. Yoon, D. W. Chan, Z.-. Q. Huang et al., "Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1," *The EMBO Journal*, vol. 22, no. 6, pp. 1336–1346, 2003.

[28] B. L. Wagner, J. D. Norris, T. A. Knotts, N. L. Weigel, and D. P. McDonnell, "The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor," *Molecular and Cellular Biology*, vol. 18, no. 3, pp. 1369–1378, 1998.

[29] X. Lan, B. S. Atanassov, W. Li et al., "USP44 is an integral component of N-CoR that contributes to gene repression by deubiquitinating histone H2B," *Cell Reports*, vol. 17, no. 9, pp. 2382–2393, 2016.

[30] P. J. Gillies, S. K. Bhatia, L. A. Belcher, D. B. Hannon, J. T. Thompson, and J. P. vanden Heuvel, "Regulation of inflammatory and lipid metabolism genes by eicosapentaenoic acid-rich oil," *Journal of Lipid Research*, vol. 53, no. 8, pp. 1679–1689, 2012.

[31] J. M. Moreno-Navarrete, F. J. Ortega, J. Bassols, W. Ricart, and J. M. Fernández-Real, "Decreased circulating lactoferrin in insulin resistance and altered glucose tolerance as a possible marker of neutrophil dysfunction in type 2 diabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 10, pp. 4036–4044, 2009.

[32] L. C. Li and R. Dahiya, "MethPrimer: designing primers for methylation PCRs," *Bioinformatics*, vol. 18, no. 11, pp. 1427–1431, 2002.

[33] F. Liu, Q. Niu, X. Fan et al., "Priming and activation of inflammasome by canarypox virus vector ALVAC via the cGAS/IFI16-STING-type I IFN pathway and AIM2 sensor," *The Journal of Immunology*, vol. 199, no. 9, pp. 3293–3305, 2017.

[34] D. Zhao, N. Zhuang, Y. Ding, Y. Kang, and L. Shi, "MiR-221 activates the NF-κB pathway by targeting A20," *Biochemical and Biophysical Research Communications*, vol. 472, no. 1, pp. 11–18, 2016.

[35] C. L. Ding, G. Xu, H. Ren et al., "HCV infection induces the upregulation of miR-221 in NF-κB dependent manner," *Virus Research*, vol. 196, pp. 135–139, 2015.

[36] H. Zheng, L. Xu, Y. Liu et al., "MicroRNA-221-5p inhibits porcine epidemic diarrhea virus replication by targeting genomic viral RNA and activating the NF-κB pathway," *International Journal of Molecular Sciences*, vol. 19, no. 11, p. 3381, 2018.

[37] C. F. Chen, J. Huang, H. Li et al., "MicroRNA-221 regulates endothelial nitric oxide production and inflammatory response by targeting adiponectin receptor 1," *Gene*, vol. 565, no. 2, pp. 246–251, 2015.

[38] C. W. Liu, H. C. Sung, S. R. Lin et al., "Resveratrol attenuates ICAM-1 expression and monocyte adhesiveness to TNF-α-treated endothelial cells: evidence for an anti-inflammatory cascade mediated by the miR-221/222/AMPK/p38/NF-κB pathway," *Scientific Reports*, vol. 7, no. 1, p. 44689, 2017.

[39] M. El Gazzar and C. E. McCall, "MicroRNAs distinguish translational from transcriptional silencing during endotoxin tolerance," *The Journal of Biological Chemistry*, vol. 285, no. 27, pp. 20940–20951, 2010.

[40] J. Yu, Y. Qiu, J. Yang et al., "DNMT1-PPARγ pathway in macrophages regulates chronic inflammation and atherosclerosis development in mice," *Scientific Reports*, vol. 6, no. 1, article 30053, 2016.

[41] S. Ogawa, J. Lozach, K. Jepsen et al., "A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1-dependent gene networks required for macrophage activation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 40, pp. 14461–14466, 2004.

[42] C. K. Glass and S. Ogawa, "Combinatorial roles of nuclear receptors in inflammation and immunity," *Nature Reviews Immunology*, vol. 6, no. 1, pp. 44–55, 2006.

[43] W. Huang, S. Ghisletti, V. Perissi, M. G. Rosenfeld, and C. K. Glass, "Transcriptional integration of TLR2 and TLR4 signaling at the NCoR derepression checkpoint," *Molecular Cell*, vol. 35, no. 1, pp. 48–57, 2009.

[44] P. Li, W. Fan, J. Xu et al., "Adipocyte NCoR knockout decreases PPARγ phosphorylation and enhances PPARγ activity and insulin sensitivity," *Cell*, vol. 147, no. 4, pp. 815–826, 2011.

[45] P. Li, N. J. Spann, M. U. Kaikkonen et al., "NCoR repression of LXRs restricts macrophage biosynthesis of insulin-sensitizing omega 3 fatty acids," *Cell*, vol. 155, no. 1, pp. 200–214, 2013.

[46] G. D. Barish, R. T. Yu, M. S. Karunasiri et al., "The Bcl6-SMRT/NCoR cistrome represses inflammation to attenuate atherosclerosis," *Cell Metabolism*, vol. 15, no. 4, pp. 554–562, 2012.

[47] P. Wiesner, S. H. Choi, F. Almazan et al., "Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor κB and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia," *Circulation Research*, vol. 107, no. 1, pp. 56–65, 2010.

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Research Article

Fluoxetine Attenuated Anxiety-Like Behaviors in Streptozotocin-Induced Diabetic Mice by Mitigating the Inflammation

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Patients with diabetes mellitus (DM) showed an increased risk of anxiety. High anxiety levels are also shown to increase stress of diabetic patients, which may contribute to poor clinical outcomes. The mechanisms underlying the development of anxiety disorders in diabetic patients remain unknown. As a result, there are no available treatments yet. Here, we tested the hypothesis that glial cells in the hippocampal area of DM mice might be responsible for their anxiety-like behaviors. Furthermore, we postulated that treatment with antidepressant, fluoxetine, could reduce anxiety behaviors and prevent the dysregulation of glial cells (oligodendrocyte and astrocyte) in DM mice. Diabetic mice were administered a single injection of streptozotocin (STZ), followed by treatment with fluoxetine. Mice were then tested on Y maze, open field, dark and light transition, and elevated plus maze tests to measure the status of anxiety and cognition. After completing these behavioral tests, mice were sacrificed and western blot was used to detect the oligodendrocyte and astrocyte maker proteins in hippocampal tissues. Emphasis was directed towards adult oligodendrocyte precursor cells (OPCs) and their marker protein to measure their proliferation and differentiation. We found that fluoxetine could effectively mitigate the level of anxiety and attenuate the cognitive dysfunction in diabetic mice. Meanwhile, fluoxetine inhibited astrocyte activation in mice exposed to STZ, prevented the loss of myelin basic protein (MBP), and affected the function of OPCs in these diabetic mice. The results suggested that the changes of these glial cells in the brains of diabetic mice might be related to the high anxiety levels and cognitive deficit in DM mice. Fluoxetine could ameliorate the high anxiety level and prevent cognitive deficit via inhibiting astrocyte activation and repairing the oligodendrocyte damage.

1. Introduction

Patients with diabetes mellitus (DM) exhibit anxiety symptoms more often than people without DM [1]. High anxiety level is not only related to poor clinical outcomes and increased complications among patients with DM, especially among elderly patients, but also greatly impacts the quality of life in DM patients [2]. Anxiety symptoms contain excessive worry, irritability, and fatigue, which contribute to the significant impairment in social functioning [3]. However, no specific treatment strategy for the disorder in DM patients has been established so far [1].

Fluoxetine (FLX) is part of a group of new-generation antidepressants called selective serotonin reuptake inhibitors (SSRIs). FLX has a better safety profile since it was shown to be safe and effective in the elderly population and even during pregnancy [4]. FLX has been widely used for treating depression and anxiety disorders in clinic [5]. Previous studies have implied that FLX could mitigate memory and cognitive deficit in animals [5]. Previous report has also indicated

that FLX could exert beneficial effects on memory and cognitive function in patients afflicted with mild cognitive impairment [6].

Recent studies have shown that myelin and oligodendrocyte deficit plays a role in the formation of depression- or anxiety-like behaviors [7, 8]. Intervention compounds targeting myelin or oligodendrocyte could effectively mitigate the depressive- and anxiety-like behaviors [7, 8]. White matter reduction was also found in the central nervous system (CNS) of patients with mood disorders [8]. Meanwhile, patients of chronic demyelinating diseases, such as multiple sclerosis (MS), demonstrated increased risk of mood disorders [9]. These findings implied that myelin deficit might play a role in the development of anxiety- and depression-like behaviors. Diabetes has been shown to be associated with myelin abnormalities such as peripheral neuropathy [10]. Diabetic peripheral neuropathy is a devastating complication in many DM patients at later stages [11]. In the CNS, a neuroimaging study also found that DM patients show alterations in metabolites of both brain gray and white matter [12]. Here, we hypothesized that glial cell abnormality in the CNS of DM mice played an important role in the deficit of memory and the formation of anxiety-like behaviors, but FLX could effectively mitigate these symptoms in DM mice by regulating the function of oligodendrocyte and astrocyte.

2. Materials and Methods

2.1. Animals and Treatments. Eight-week-old male mice of C57BL/6J were used in the study. The mice were kept in standard environment for laboratory animals. All the mice were given one-week acclimation period before any experimental procedure was carried on. Animal protocols were approved by the Animal Care Committee of Qingdao Municipal Hospital, Shandong, China.

Four groups of mice were used in the present study: control ($n = 10$), control plus FLX (5 mg/kg/day; $n = 10$), STZ (150 mg/kg, $n = 10$), and STZ plus FLX (5 mg/kg/day; $n = 10$). STZ and FLX were purchased from Sigma-Aldrich (MO, USA) and Santa Cruz Biotechnology (Dallas, TX). STZ was prepared in distilled sodium citrate solution with pH at 4.5 and FLX was dissolved in sterile water (2 mg/100 ml water). Single-dose intraperitoneal injection of STZ was administrated to cause DM in a mouse model as previously reported [13]. Water containing FLX was given to mice one week ahead of the STZ injection and lasted till the last day of behavioral test. Behavioral tests were performed 3 weeks later after the STZ injection. Body weight of mice was measured twice per week. We sacrificed the mice and collected the brain samples immediately after the behavioral tests were finished.

2.2. Open Field. A square box was used for performing the open field test as previously described with minor modification [14]. Briefly, mice were put in a corner of the open field before the test was initiated. The activity of each mouse in the central and peripheral areas was observed. The total time in the central area and distance travelled during the whole test were analyzed.

2.3. Elevated Plus Maze. An elevated plus-shaped maze (EPM) with two open and two closed arms (45 cm × 10 cm) was used for this experiment. The height of the maze to the ground was about 50 cm (elevated). Before starting the test, we put the mice in the central area by facing an open arm. During the test, mice were allowed to probe the elevated maze for 5 min [15]. When four paws of the mouse entered the arms, a valid entry was considered and recorded. The total time of each mouse which stayed in the open arms and the number of each mice entering the total open arms in each test session were recorded for statistical analysis.

2.4. Dark and Light Transition Test. Dark and light transition test was carried out as previously described [16]. The device is divided into a light and dark box and there is a shuttle door between these two boxes. The walls of the dark box were painted black with a removable black lid on the top. Mouse was put in the dark box covered with the lid and the shuttle door was closed. After 1 minute, the shuttle door was opened to allow the mouse to freely go into the light box. We counted the first latency of the mouse entering the light box as well as the numbers of transitions between the two boxes. The duration of the test was 5 minutes.

2.5. Y Maze. The Y maze spontaneous alternation test is a behavioral test to investigate the willingness of mice to probe novel environments. The apparatus consisted of 3 arms diverging at a 120° from the central point. The procedure was carried out as previously reported with minor change [5]. Each mouse was initially put at the end of one arm by facing all and was allowed to probe the Y maze during an eight-minute test session. The sequences of entering the arms and the number of total entries during the period of 8 minutes were recorded. Percentage of alternation was expressed as the number of sequential triplets containing entries in the three arms during the session as a proportion of the maximum possible alternation (equivalent to the number of total entries minus 2) × 100.

2.6. Western Blot. The extracted brain hippocampal samples were run by electrophoresis with SDS-PAGE gel. And then, the protein samples were transferred onto nitrocellulose membranes. We blocked the above membranes with 5% skim milk dissolved in TBST buffer and then incubated the membranes with the primary antibodies against the interested proteins, including antibody to glial fibrillary acidic protein (GFAP) (Millipore Corporation, MA, USA), antibody to platelet-derived growth factor receptor-a (PDGFRα) (Santa Cruz Biotechnology), anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (Millipore Corporation, MA, USA), and myelin basic protein (MBP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for overnight at 4°C. After being washed 3 times, the above membranes were incubated with their corresponding secondary antibodies for another 2 hours under room temperature. We visualized the bands in the membranes by chemiluminescence reaction (Amersham Biosciences, NJ, USA). GAPDH (Abcam, UK) or β-actin (Abcam, UK) antibodies were used to detect the protein expression of these housekeeping genes and considered as

the loading controls. Results of each protein were expressed as a ratio of it compared to internal control protein.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). ELISA kits (eBioscience, Thermo Fisher Scientific) were used for the investigation of IL-6 level in brain tissues according to the manufacturer manuals. Samples were loaded into plate wells in duplicate, and the average of two values was used for final analysis. The final value of IL-6 in statistic was expressed as a ratio of their averages compared to total loading protein.

2.8. Statistical Analysis. Values in this study were expressed as the mean ± SEM. The significant differences were defined with one-way ANOVA, followed by Newman-Keuls *post hoc* test. We considered a p value of less than 0.05 as statistically significant.

3. Results

3.1. FLX Improved the Working Memory Performance of DM Mice in a Y Maze Test. DM mice reportedly showed significant deficits on memory and cognitive dysfunction [17]. Therefore, we evaluated the FLX influence on the working memory of DM mice by performing a Y maze test. DM mice exerted significant decrease on the spontaneous alternation (Figure 1(a)), which suggested deficits of working memory. However, FLX could effectively prevent the memory loss of DM mice (Figure 1(a)) ($F_{(3,36)} = 5.734$, STZ group compared to the control group, $^{*}p < 0.01$; $F_{(3,36)} = 5.734$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). Additionally, our results also demonstrated that no significant difference existed on total arm entries between groups (Figure 1(b)), which implicated that the difference on the alternation between groups was not due to the changes of mobility of mice.

3.2. FLX Attenuated the Anxiety-Like Behaviors of DM Mice. Next, we measured the anxiety-like behaviors in these DM mice and investigated whether FLX could attenuate these abnormal behaviors. Open field test was employed here to measure the level of anxiety in mice. In this study, the total travel distance and total time spent in the center of each mouse were recorded for the analysis (Figure 2). We found that the DM mice traveled less distance and spent less time in the central area of the open field. Interestingly, mice under FLX treatment showed obvious better performance in distance ($F_{(3,36)} = 10.13$, STZ group compared to the control group, $^{*}p < 0.001$; $F_{(3,36)} = 10.13$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA) and time in the center ($F_{(3,36)} =$, STZ group compared to the control group, $^{*}p < 0.05$; $F_{(3,36)} = 3.465$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). We also used EPM to test the anxiety level of these DM mice. As shown in Figure 3(a), FLX effectively increased the time spent in the open arms of DM mice in the EPM test, which implied that FLX ameliorated anxiety-like behaviors in the EPM test too ($F_{(3,36)} = 3.695$, STZ group compared to the control group, $^{*}p < 0.05$; $F_{(3,36)} = 3.695$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). These effects were further validated by the following parameters shown in Figure 3(b) that FLX increased the total numbers of entering the open arms in DM mice ($F_{(3,36)} = 4.913$, STZ group compared to the control group, $^{*}p < 0.01$; $F_{(3,36)} = 3.695$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). Lastly, light and dark transition test was introduced to further confirm the effects of FLX on the anxiety-like behaviors of these DM mice. Consistent with the results above, FLX prevented the reduction of time spent in the light box of DM mice (Figure 4(a)) ($F_{(3,36)} = 5.998$, STZ group compared to the control group, $^{*}p < 0.01$; $F_{(3,36)} = 5.998$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). Meanwhile, FLX reduced the latency of DM entering the light box for the first time during the test (Figure 4(b)) ($F_{(3,36)} = 3.713$, STZ group compared to the control group, $^{*}p < 0.05$; $F_{(3,36)} = 3.713$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). Collectively, all the findings in these above tests implied that FLX could be an effective compound to attenuate the anxiety-like performances and memory loss in DM mice.

3.3. FLX Decreased the Levels of GFAP and IL-6 but Increased the MBP, PDGFRα, and CNPase Protein Levels of DM Mice. Next, we explored the possible underlying mechanism by which FLX protected DM mice against memory loss and anxiety-like behavior formation. To test whether astrocyte activation was involved in the regulation of FLX on mice exposed to STZ, western blot assays were firstly used with antibody of GFAP, which is a common protein maker of astrocyte activation. As shown in Figure 5(a), the GFAP expression level was increased in STZ-induced DM mice, which could be prevented by the cotreatment of FLX. Meanwhile, we found that the level of IL-6 was significantly upregulated in the brain samples of STZ mice but could be inhibited by FLX treatment (Figure 5(e)) ($F_{(3,22)} = 10.31$, STZ group compared to the control group, $^{*}p < 0.05$; $F_{(3,22)} = 10.31$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; one-way ANOVA). These two results suggested that FLX could ameliorate the activation of astrocyte in the brain exposed to STZ. Secondly, we investigated whether STZ caused myelin damage in the hippocampal area of the DM mice by detecting the MBP expression level. We found that there was notable reduced expression level of MBP in DM mice but FLX could ameliorate the MBP loss (Figure 5(b)). Thirdly, we asked whether FLX affected the adult OPCs, which usually surround the lesion area of myelin and play important roles during the myelin repair in many pathological scenarios [8, 18]. We focused on two proteins, CNPase and PDGFRα. CNPase is an important protein during the development of oligodendrocytes to become myelin-forming cells [19]. PDGFRα is one of the feature protein markers of OPCs [20]. As shown in Figures 5(c) and 5(d), STZ treatment led to significant reduction of PDGFRα ($F_{(3,22)} = 3.31$, STZ group compared to the control group, $^{*}p < 0.05$; one-way ANOVA) and CNPase ($F_{(3,36)} = 4.094$,

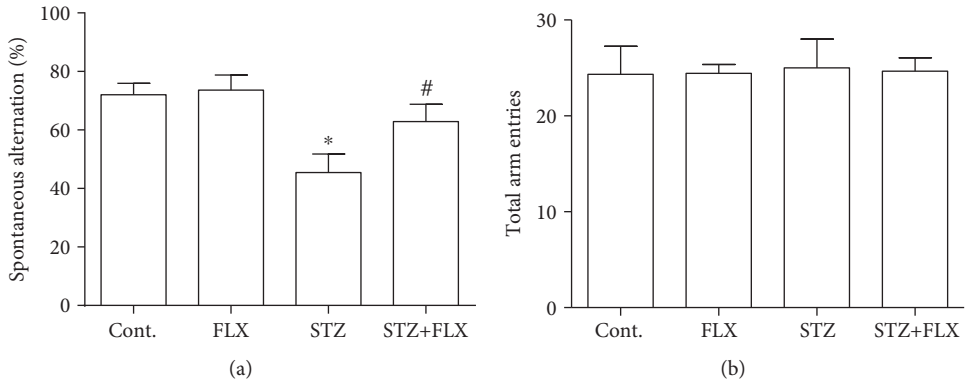

Figure 1: FLX attenuated spatial working memory impairment of DM mice in a Y maze test. (a) STZ induced significant decreased spontaneous alternation of DM mice but FLX could prevent the decrease in DM mice on the working memory performance. (b) The total arm entries among all groups were not influenced by STZ or FLX. All values were expressed as means ± SEM. $n = 10$. $^{*}p < 0.01$ compared to the control group; $^{\#}p < 0.05$ compared to the STZ group.

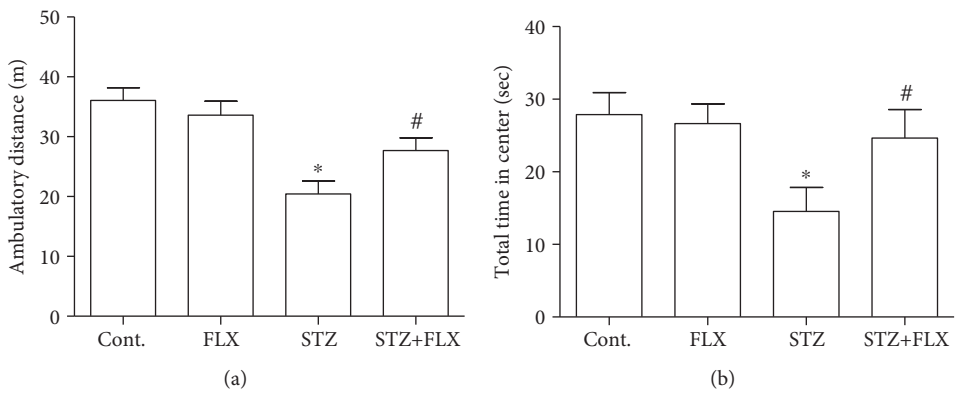

Figure 2: FLX attenuated anxiety-like behaviors of DM mice in the open field. (a) FLX improved the total travel distance of DM mice in the test duration. (b) FLX increased the total time spent of DM mice at the center area. All values were expressed as means ± SEM. $n = 10$. $^{*}p < 0.05$ or 0.01 compared to the control group; $^{\#}p < 0.05$ compared to the STZ group.

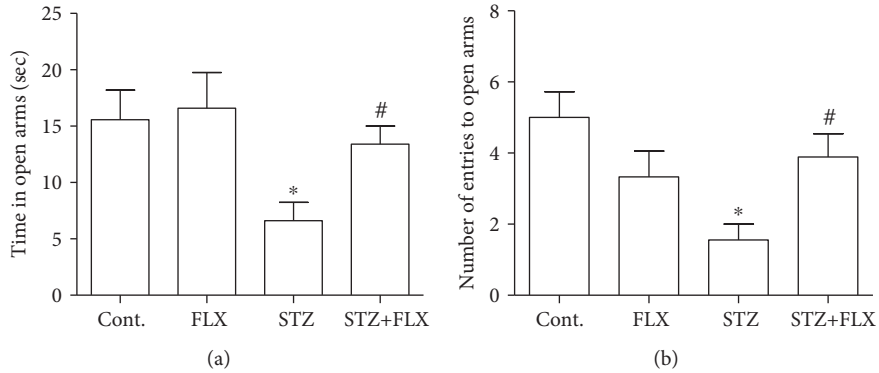

Figure 3: FLX attenuated anxiety-like behaviors of DM mice in an elevated plus maze test. (a) FLX increased the total time spent of DM mice at the open arms. (b) FLX increased the total number of DM mice entering the open arms. All values were expressed as means ± SEM. $n = 10$. $^{*}p < 0.05$ or 0.01 compared to the control group; $^{\#}p < 0.05$ compared to the STZ group.

STZ group compared to the control group, $^{*}p < 0.05$; one-way ANOVA) expression but FLX prevented the protein loss of both these proteins, respectively ($F_{(3,22)} = 3.31$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$; $F_{(3,36)} = 4.094$, STZ+FLX group compared to the STZ alone group, $^{\#}p < 0.05$). Our results indicated that FLX might

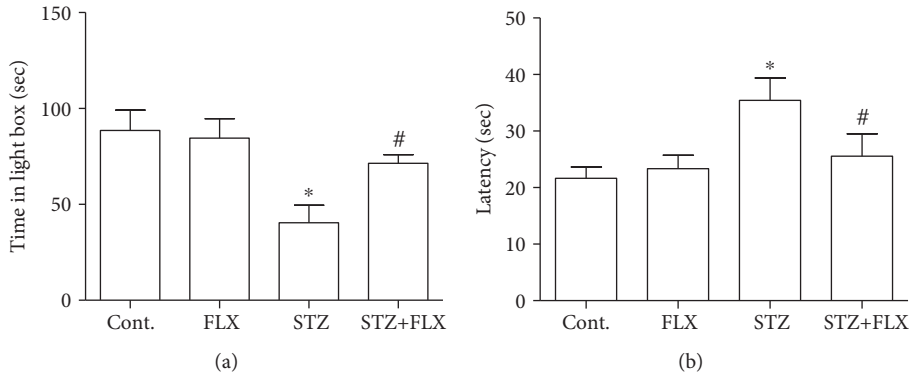

Figure 4: FLX attenuated anxiety-like behaviors of DM mice in the dark and light transition test. (a) FLX increased the total time spent of DM mice at the light box. (b) FLX shortened the latency time of DM mice entering the light box for the first time during the test. All values were expressed as means ± SEM. $n = 10$. $^{*}p < 0.05$ or 0.01 compared to the control group; $^{\#}p < 0.05$ compared to the STZ group.

improve the behavioral performance in DM mice by inhibiting the glial cell activation and protect myelin and OPC loss in the hippocampal area of DM mice.

4. Discussion

Recent findings support that the brain is one of the common targets of diabetic complications [21]. The possibility of mood disorders such as depression in patients with DM is doubled than that in patients without DM [22]. Moreover, patients with DM cooccurring with anxiety are at an increased risk of mortality [23]. Anxiety is closely relevant with the dysregulation of the hypothalamic-pituitary-adrenal axis (HPA), which can trigger insulin resistance [24, 25]. Therefore, anxiety or other mood disorders may be a contributing factor for DM through biological and behavioral manners. So far, there are no specific compounds available clinically to treat anxiety or depression in DM patients. Here, we used a STZ-induced DM mouse model to investigate whether FLX could effectively prevent the formation of anxiety-like behaviors and memory deficit in DM mice. We found that DM mice showed obvious anxiety performances in a series of behavioral tests. The results here are consistent with reports from other groups [26, 27]. More importantly, our results also disclosed that FLX treatment could mitigate these abnormal behavioral performances. In the Y maze test, STZ induced significant decreased spontaneous alternation but could be reversed by FLX treatment (Figure 1). These results suggested that FLX not only could protect DM patients against the formation of anxiety-like behaviors but also exert protective effects on memory and cognitive deficit.

Astrocyte activation is reportedly found in the brain of DM mice [27]. Here, we found that astrocyte activation maker protein, GFAP, was notably upregulated on the protein level (Figure 5(a)), which suggested a neuroinflammatory response in the CNS of DM mice. Interestingly, the increase of GFAP was inhibited by FLX cotreatment. At the same time, the level of IL-6 in the mouse brain was increased but could be inhibited by the FLX treatment. These results were consistent with a previous report that FLX could inhibit the activation of astrocytes in animal models of Alzheimer's disease [28]. And then, we explored the beneficial effects of FLX on oligodendrocyte by detecting the myelin protein, MBP. We found that there was an improvement of MBP protein expression accompanying the inhibition of GFAP (Figure 5(b)). These results reminded us to look at another glial cell, oligodendrocyte. Adult OPC proliferation and differentiation are among the key features of myelin regeneration [29]. Two important marker proteins of OPCs were investigated with western blot in the studies that followed. We found that STZ caused reduced expression of PDGFRα and CNPase, which could be reversed by FLX (Figures 5(c) and 5(d)). These results suggested that FLX could also regulate the adult OPCs in the hippocampal area of DM mice and improve their behavioral performance.

Neurobehavioral complication in DM patients is a rising problem that needs research attention since the damage to the neurosystem has a significant impact on the medical cost and life quality of DM patients. According to the knowledge we have known so far, there are fewer efforts made to the preclinical setting about this issue. Our study provided evidences that anxiety and memory loss are the essential part of neuropathological changes of DM mice. And the results were in line with a previous report that anxiety-like behaviors could develop in mice with STZ exposure [30]. FLX may be a good candidate to handle these disorders in DM patients by attenuating the astrocyte activation and rescuing the myelin deficit in the CNS. Meanwhile, the beneficial effects could also result from the direct anti-inflammatory role since FLX already was shown to be able to inhibit the inflammatory response from astrocytes [28]. Further studies involving the other inflammatory cells in the CNS, like microglia, are needed to elaborate the details of cellular response in STZ-induced DM mice.

5. Conclusions

The principal finding of this study is that FLX was able to exert its preventive effects on memory loss and anxiety behaviors in DM mice. The beneficial effects of FLX may be

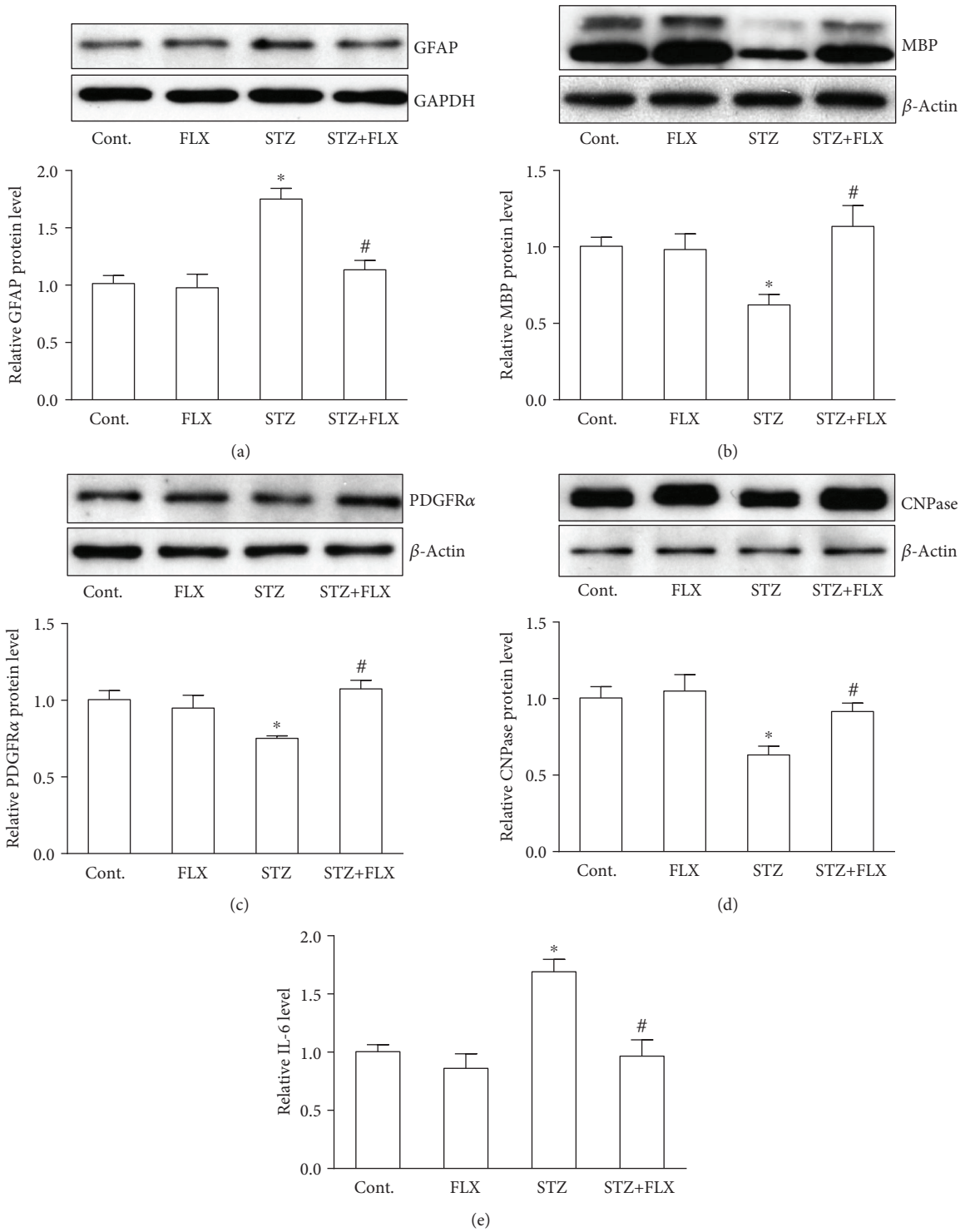

Figure 5: FLX influenced the protein expressions of astrocytes and oligodendrocytes in the CNS of DM mice. (a) FLX reduced the expression level of GFAP. (b) FLX increased the expression level of MBP. (c) FLX increased the expression level of PDGFRα. (d) FLX increased the expression level of CNPase. (e) FLX reduced the expression level of IL-6. All values were expressed as means ± SEM. $n = 5$. $^{*}p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the STZ group.

due to its regulation on astrocyte activation and oligodendrocyte dysfunction in the CNS of DM mice. Whether FLX can provide benefit to patients suffering from DM warrants further investigation in clinical setting.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

No conflicts of interest exist for all authors in this study.

Acknowledgments

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References

[1] A. Bickett and H. Tapp, "Anxiety and diabetes: innovative approaches to management in primary care," *Experimental Biology and Medicine*, vol. 241, no. 15, pp. 1724–1731, 2016.

[2] D. Kohen, A. P. Burgess, J. Catalán, and A. Lant, "The role of anxiety and depression in quality of life and symptom reporting in people with diabetes mellitus," *Quality of Life Research*, vol. 7, no. 3, pp. 197–204, 1998.

[3] A. K. Khuwaja, S. Lalani, R. Dhanani, I. S. Azam, G. Rafique, and F. White, "Anxiety and depression among outpatients with type 2 diabetes: a multi-centre study of prevalence and associated factors," *Diabetology & Metabolic Syndrome*, vol. 2, no. 1, 2010.

[4] A. Rossi, A. Barraco, and P. Donda, "Fluoxetine: a review on evidence based medicine," *Annals of General Hospital Psychiatry*, vol. 3, no. 1, p. 2, 2004.

[5] D. S. Sun, L. F. Gao, L. Jin et al., "Fluoxetine administration during adolescence attenuates cognitive and synaptic deficits in adult 3×TgAD mice," *Neuropharmacology*, vol. 126, pp. 200–212, 2017.

[6] A. Mowla, M. Mosavinasab, H. Haghshenas, and A. B. Haghighi, "Does serotonin augmentation have any effect on cognition and activities of daily living in Alzheimer's dementia?: a double-blind, placebo-controlled clinical trial," *Journal of Clinical Psychopharmacology*, vol. 27, no. 5, pp. 484–487, 2007.

[7] Y. Yamazaki, Y. Hozumi, K. Kaneko et al., "Modulatory effects of oligodendrocytes on the conduction velocity of action potentials along axons in the alveus of the rat hippocampal CA1 region," *Neuron Glia Biology*, vol. 3, no. 4, pp. 325–334, 2007.

[8] V. M. Vostrikov, N. A. Uranova, and D. D. Orlovskaya, "Deficit of perineuronal oligodendrocytes in the prefrontal cortex in schizophrenia and mood disorders," *Schizophrenia Research*, vol. 94, no. 1-3, pp. 273–280, 2007.

[9] P. A. Arnett, F. H. Barwick, and J. E. Beeney, "Depression in multiple sclerosis: review and theoretical proposal," *Journal of the International Neuropsychological Society*, vol. 14, no. 5, pp. 691–724, 2008.

[10] G. Cermenati, F. Abbiati, S. Cermenati et al., "Diabetes-induced myelin abnormalities are associated with an altered lipid pattern: protective effects of LXR activation," *Journal of Lipid Research*, vol. 53, no. 2, pp. 300–310, 2012.

[11] A. Gordois, P. Scuffham, A. Shearer, A. Oglesby, and J. A. Tobian, "The health care costs of diabetic peripheral neuropathy in the U.S.," *Diabetes Care*, vol. 26, no. 6, pp. 1790–1795, 2003.

[12] S. C. Ferguson, A. Blane, J. Wardlaw et al., "Influence of an early-onset age of type 1 diabetes on cerebral structure and cognitive function," *Diabetes Care*, vol. 28, no. 6, pp. 1431–1437, 2005.

[13] M. C. Deeds, J. M. Anderson, A. S. Armstrong et al., "Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models," *Laboratory Animals*, vol. 45, no. 3, pp. 131–140, 2011.

[14] S. Akter, H. Sasaki, K. R. Uddin, Y. Ikeda, H. Miyakawa, and S. Shibata, "Anxiolytic effects of γ-oryzanol in chronically-stressed mice are related to monoamine levels in the brain," *Life Sciences*, vol. 216, pp. 119–128, 2019.

[15] M. Fréchou, I. Margaill, C. Marchand-Leroux, and V. Beray-Berthat, "Behavioral tests that reveal long-term deficits after permanent focal cerebral ischemia in mouse," *Behavioural Brain Research*, vol. 360, pp. 69–80, 2019.

[16] M. Umemura, T. Ogura, A. Matsuzaki et al., "Comprehensive behavioral analysis of activating transcription factor 5-deficient mice," *Frontiers in Behavioral Neuroscience*, vol. 11, p. 125, 2017.

[17] S. C. Fang, H. Xie, F. Chen et al., "Simvastatin ameliorates memory impairment and neurotoxicity in streptozotocin-induced diabetic mice," *Neuroscience*, vol. 355, pp. 200–211, 2017.

[18] C. Takahashi, R. Muramatsu, H. Fujimura, H. Mochizuki, and T. Yamashita, "Prostacyclin promotes oligodendrocyte precursor recruitment and remyelination after spinal cord demyelination," *Cell Death & Disease*, vol. 4, no. 9, article e795, 2013.

[19] G. Garthwaite, K. Hampden-Smith, G. W. Wilson, D. A. Goodwin, and J. Garthwaite, "Nitric oxide targets oligodendrocytes and promotes their morphological differentiation," *Glia*, vol. 63, no. 3, pp. 383–399, 2015.

[20] A. R. Calver, A. C. Hall, W. P. Yu et al., "Oligodendrocyte population dynamics and the role of PDGF in vivo," *Neuron*, vol. 20, no. 5, pp. 869–882, 1998.

[21] R. J. Anderson, K. E. Freedland, R. E. Clouse, and P. J. Lustman, "The prevalence of comorbid depression in adults with diabetes: a meta-analysis," *Diabetes Care*, vol. 24, no. 6, pp. 1069–1078, 2001.

[22] K. Naicker, J. A. Johnson, J. C. Skogen et al., "Type 2 diabetes and comorbid symptoms of depression and anxiety: longitudinal associations with mortality risk," *Diabetes Care*, vol. 40, no. 3, pp. 352–358, 2017.

[23] B. E. Cohen, P. Panguluri, B. Na, and M. A. Whooley, "Psychological risk factors and the metabolic syndrome in patients with coronary heart disease: findings from the Heart and Soul Study," *Psychiatry Research*, vol. 175, no. 1-2, pp. 133–137, 2010.

[24] I. Chiodini, G. Adda, A. Scillitani et al., "Cortisol secretion in patients with type 2 diabetes: relationship with chronic complications," *Diabetes Care*, vol. 30, no. 1, pp. 83–88, 2007.

[25] J. P. Damián, V. Acosta, M. da Cuña et al., "Effect of resveratrol on behavioral performance of streptozotocin-induced diabetic mice in anxiety tests," *Experimental Animals*, vol. 63, no. 3, pp. 277–287, 2014.

[26] A. N. Sharma, K. M. Elased, T. L. Garrett, and J. B. Lucot, "Neurobehavioral deficits in *db*/*db* diabetic mice," *Physiology & Behavior*, vol. 101, no. 3, pp. 381–388, 2010.

[27] F. E. Saravia, Y. Revsin, M. C. Gonzalez Deniselle et al., "Increased astrocyte reactivity in the hippocampus of murine models of type 1 diabetes: the nonobese diabetic (NOD) and streptozotocin-treated mice," *Brain Research*, vol. 957, no. 2, pp. 345–353, 2002.

[28] J. Qiao, J. Wang, H. Wang et al., "Regulation of astrocyte pathology by fluoxetine prevents the deterioration of Alzheimer phenotypes in an APP/PS1 mouse model," *Glia*, vol. 64, no. 2, pp. 240–254, 2016.

[29] M. Podbielska, N. Banik, E. Kurowska, and E. Hogan, "Myelin recovery in multiple sclerosis: the challenge of remyelination," *Brain Sciences*, vol. 3, no. 4, pp. 1282–1324, 2013.

[30] D. Gupta, M. Radhakrishnan, and Y. Kurhe, "Insulin reverses anxiety-like behavior evoked by streptozotocin-induced diabetes in mice," *Metabolic Brain Disease*, vol. 29, no. 3, pp. 737–746, 2014.

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Research Article

Propofol Induces Postoperative Depression and Inhibits Microglial Function in Mice

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Many patients experience excellent physical recoveries after surgery; however, there are some of them who from suffer mood fluctuation, even depression. Postoperative depression may be resulted from cognitive dysfunction, pain, and a compromised immune system during the surgery. But there is a higher possibility that general anaesthesia may be responsible for the development of depression. Here, we employed one of the most used anaesthetics, propofol, in a mouse model to investigate whether this intravenous anaesthetic compound could cause depressive-like behavioural performance in mice. We found a single dose of propofol caused significant abnormal behavioural performance in tail suspension, forced swimming, and open field tests. We also examined the brain section of these mice and revealed that there was significant reduced expression of the CD11b protein, which demonstrated an inhibition of propofol on microglial function. We investigated the effect of propofol on synaptic protein, SYP, and found there was no notable influence on the protein expression. These above results suggested that propofol treatment might promote the depressive-like behaviours in mice via influencing the microglial cell function. Furthermore, we found the level of the IL-6 cytokine was significantly increased in the brain tissue, which might subsequently cause the activation of the transcriptional factor, STAT3. Our finding may provide a new perspective of further understanding the mechanism of anaesthetic drugs and deciphering the underlying mechanism of postoperative depression.

1. Introduction

Many patients who undergo general anaesthesia or surgery experience some form of postsurgical depression, especially during the six months following an invasive procedure [1]. As one of the frequent complications after surgery, depression may lead to further morbidity and mortality, especially for elderly patients [1]. Meanwhile, researchers have discovered that depressed patients are more likely to have other complications after surgery [2]. They are less able to cooperate well with the caregivers in their after-surgery care, such as rehabilitative therapy. In patients who already have a preexisting depression or anxiety history, the recovery time from postsurgical depression is much longer [3]. A study also suggested surgery might exacerbate the severity of the preexisting depression [4]. So far, little is known about why there is such a strong link between surgery and depression. Some researchers have thought the psychological reason per se is that many people experience postsurgical depression because the surgical procedures force them to confront their own mortality. Some studies also emphasized the specific types of surgery per se on the induction of postsurgical depression since depression seems to be more often observed in some major surgeries, including brain surgery, hip replacement surgery, and cancer resection. However, recent studies suggested that the length of time spent under anaesthesia seemed to be related to the likelihood and severity of depression [5]. Propofol is one of the most used anaesthetics in the intensive care setting after surgery [6]. Propofol treatment has been found to be significantly associated with cognitive dysfunctions in the postoperative period [7]. There is no effort made to examine whether and how propofol could impact the mood status in patients and animals so far. Therefore, we employed a mouse model with a single dose of propofol

treatment and tested the depressive-like behaviours in the mouse model. We found mice exposed to a single dose of propofol treatment exerted anxiety-like behaviours in an open field test by showing less time in the center area and depressive-like behaviours in tail suspension and forced swimming tests by showing longer immobility time than control mice without injection of propofol. Our results indicated that propofol might be responsible for the mood fluctuation after surgery and this effect may be associated with the influence of propofol on the microglia cells in the central nervous system (CNS).

2. Materials and Methods

2.1. Animals and Drug Treatment. In the present study, we used 8-10 weeks old male C57BL/6 mice. All mice were free to access water and food. For all the animal studies here, mice were treated strictly following the guidelines established by the Chinese Council on Animal Care. All the procedures were approved by the Animal Care Committee of Qingdao Municipal Hospital, Shandong, China. There were two groups of mice in the study: normal saline (controls; $n = 10$) and propofol (75 mg/kg; $n = 10$). A single-dose injection of propofol was administrated to the mice intraperitoneally. Commercial propofol injection solution (Xi'an Libang Pharmaceuticals, China) was used here. The dose of propofol used here is adapted from the previous study [8]. During the anaesthesia time, all mice were put on the heating pad to maintain their body temperature. Behavioural tests were performed 1 week later.

2.2. Open Field. A square wood box was used here for the open field test as previously described [9]. At the beginning of the test, each mouse was placed in a corner of the box facing the wall. The total traveled distance and total time spent in the inner squares of all mice were recorded and measured in a 5-minute session.

2.3. Tail Suspension Test. For the tail suspension test, the procedures were performed as previously reported [10]. Mice were suspended through tails with a tape on a small metal hook, and they cannot escape or hold on to nearby surfaces in this position. The total time the mice spent immobile during the 6 min testing period was recorded. Immobility is defined as a lack of attempt of mice to move their bodies.

2.4. Forced Swimming Test. We carried out the forced swimming test as previously reported [11]. Mice were placed in a glass beaker filled with water at room temperature. We tested the total immobile time of mice in a 15 min testing session, and the last 6 min was scored for immobility duration. At the end of each test, the wet mice were immediately placed in a cage with normal bedding after they were warmed up in a dry towel.

2.5. Elevated Plus Maze Test. The elevated plus maze is a simple method for evaluating animal anxiety responses. We tested the anxiety level of mice by using the elevated plus maze assay as previously reported [12]. Basically, the elevated plus maze apparatus is equipped with two open and two closed arms and elevated to a height of around 50 cm above the ground. At the beginning of each test, the mouse was placed in the central square by facing the open arm and then was allowed to explore the arms for 5 min. The amount of time spent in the open arms was recorded and analyzed by a person who was not involved in the experimental design.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). We measured the IL-6 level in brain tissues by using a commercial ELISA kit (eBioscience, Thermo Fisher Scientific). Each sample was assayed in a duplicate manner with appropriate dilutions in order that relative luminescent units could fall within the linear range of standard curves. The value of IL-6 from each sample was normalized and expressed as a ratio compared to the total loading protein as a relative ratio. The absorbance of each sample was measured with a microplate reader (Synergy Mx, BioTek, Winooski, VT).

2.7. Western Blot. Dissolved brain samples were processed and run on SDS-PAGE gels. They were then transferred onto PVDF membranes that were then blocked with 5% skim milk in TBST buffer. The blocked membranes were further investigated with antibodies to CD11b (1 : 4000; Abcam, UK), synaptophysin (SYP), p-Stat-3 (1 : 1000; Cell Signaling, Danvers, MA), and total Stat-3 (1 : 1000; Cell Signaling, Danvers, MA) in TBST milk overnight at 4°C. After incubation with the secondary antibodies for 2 hours at room temperature respectively, the bands of protein on the membrane were disclosed with chemiluminescence reaction. β-Actin was used as an internal control (1 : 5000; Santa Cruz Biotechnology, CA, USA). Quantitative results were expressed as a ratio of each target protein to its β-actin.

2.8. Statistical Analysis. Values presented in the study were shown as the mean ± SEM. The significance of difference between two groups was determined by Student's t-test analysis. A p value of less than 0.05 was regarded as statistically significant.

3. Results

3.1. Single Dose of Propofol Exposure Caused Depressive-Like Behaviours in Mice with Tail Suspension and Forced Swimming Tests. Firstly, we tested the hypothesis whether a single dose of propofol treatment could cause long-term effects on the behavioural performance in mice by focusing on the depression manner. We employed the tail suspension and forced swimming tests in these mice to explore the long-term effects of propofol. In the tail suspension test, we found propofol significantly increased the immobile time compared to the control mice without propofol treatment when the mice were hanged in tails (Figure 1). To further confirm the depressive-like behaviours in the mice exposed to propofol, we used the force swimming method to measure the immobile time of these mice. Our results demonstrated that propofol increased the total time of immobile time compared to mice in the control group (Figure 2). These above findings revealed that a single dose of propofol treatment could cause and sustain the long-term depressive behavioural performance in mice.

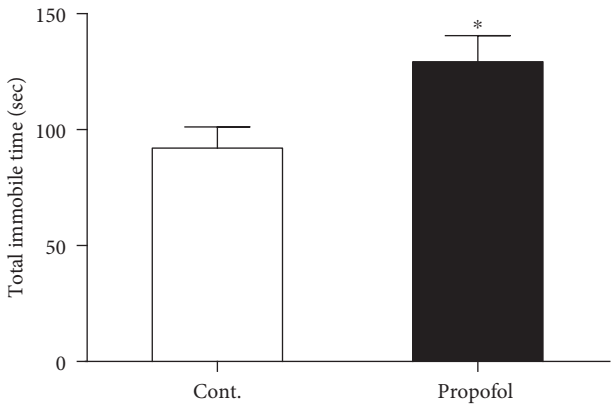

Figure 1: Propofol increased the total immobile time of mice compared to control mice in the tail suspension test. All data are expressed as the means ± SEM. $n = 10$. $^*p < 0.05$.

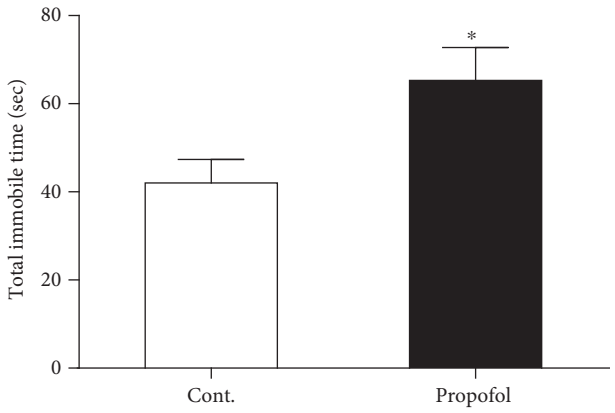

Figure 2: Propofol increased the total immobile time of mice compared to control mice in the forced swimming test. All data are expressed as the means ± SEM. $n = 10$. $^*p < 0.05$.

3.2. Single Dose of Propofol Exposure Caused Anxiety-Like Behaviours in Mice with Open Field and Elevated Plus Maze Tests. Anxiety is the highest cooccurrence complication in depression. Therefore, we postulated whether propofol could induce anxiety-like behaviours in these mice, basing on the above behavioural results in these mice. We used the open field assay to test the anxiety level of these mice. As shown in Figure 3(a), propofol treatment effectively reduced the total time mice spent on the center area in the open field. And we also measured another important parameter of the test, the total travel distance of the mice. As we expected, propofol injection significantly decreased the travel distance of mice in the 5 min test session compared to control mice (Figure 3(b)). Next, we investigated the anxiety-like behaviours of these mice in elevated plus maze assay. We found that mice exposed to propofol showed significant less time spent in open arms (Figure 4), which was in line with the results from the open field test.

3.3. Single Dose of Propofol Exposure Caused the Increased Level of IL-6 in the Brain Tissues of Mice. A recent study suggested the involvement of neuroinflammation in postoperative delirium-like cognitive deficits [13]. We tested whether propofol could impact the expression level of IL-6 in the brain tissues of these mice. To achieve the conclusion, we performed ELISA assay in the brain tissues (hippocampus and cortex) to assess the level of IL-6 in mice with or without propofol treatment. Our results demonstrated that propofol treatment in this condition could be able to upregulate the expression level of this cytokine in the brains of mice exposed to propofol (Figure 5). This result indicated that propofol might cause the anxiety- and depressive-like behaviours in mice by affecting the inflammatory response in their brains.

3.4. Single Dose of Propofol Exposure Caused the Reduced Expression of CD11b and Increased Expression of p-STAT-3 in the Brain Tissues of Mice. Last, we probed the possible cellular and molecular mechanisms that might be responsible for the behavioural changes in these mice exposed to propofol. We tested whether glial cells were influenced by the propofol treatment by looking at the microglial maker protein, CD11b. With a western blot study, we found the expression level of CD11b was reduced in the brains of mice with propofol treatment (Figure 6(a)), which suggested that microglial function might be regulated by the propofol treatment. Meanwhile, the expression of the presynaptic protein SYP was not affected by the propofol treatment (Figure 6(c)). We also studied the function of the transcriptional factor STAT-3 by investigating the phosphorylation status of STAT-3 (p-STAT-3). Our western blot results demonstrated that propofol treatment caused the increased expression level of p-STAT-3 without affecting the expression of total STAT-3 (Figure 6(b)). These findings implicated that propofol might influence the microglial cell function and enhance the phosphorylation of STAT-3 while inducing the anxiety- and depressive-like behavioural performances in mice.

4. Discussion

Cognitive and memory dysfunctions have been fairly studied, but the postoperative mood fluctuation has not attracted enough attention so far. Some evidences supported the idea that there were significant mood changes that occurred in the patients who are exposed to surgery and anaesthetic treatment. Here, we tested a new hypothesis that anaesthetic treatment per se may be enough to significantly affect the mood status in animals that underwent the single dose of anaesthetic treatment without an accompanying surgical procedure. We used propofol as the representative anaesthetic compound and intraperitoneally injected it to the mice. In the following days, a series of depressive and anxiety behaviours were performed to observe the mood changes in the mice. We found that there were significant differences on the behavioural performances between these mice with or without propofol exposure. Since these propofol-induced effects were sustained for a significant time while even after the medication was took off, we postulated that these effects were not the acute anaesthetic influence but were mediated by other systems in the CNS.

Neuroinflammation has been found to be one of the major factors that contribute to the depression and other mood changes in the CNS [14]. Here, we asked whether a

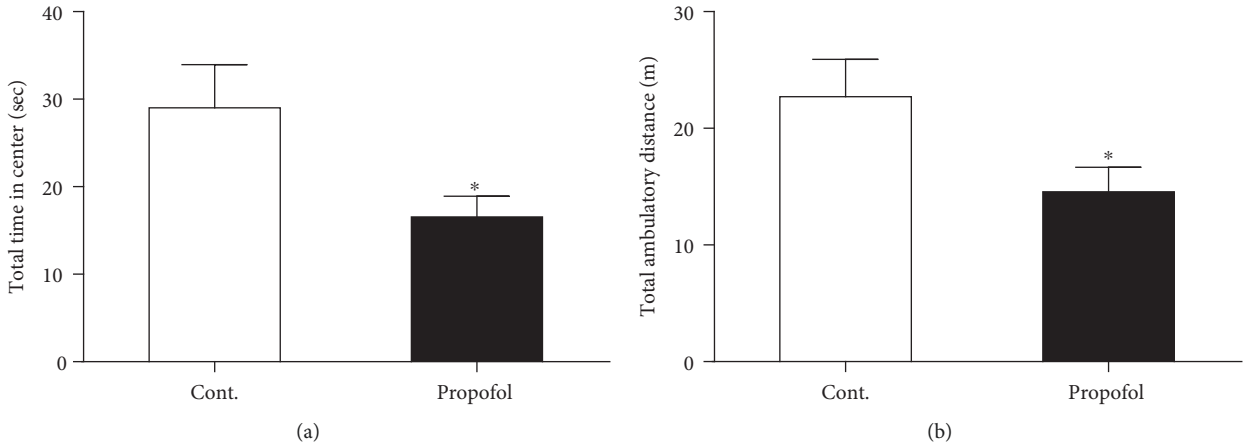

Figure 3: Propofol caused anxiety-like behaviours of mice in the open field test. (a) Propofol decreased the total time in the center area of mice compared to control mice in the open field test. (b) Propofol decreased the total travel distance of mice compared to control mice in the open field test. All data are expressed as the means ± SEM. $n = 10$. $^{*}p < 0.05$.

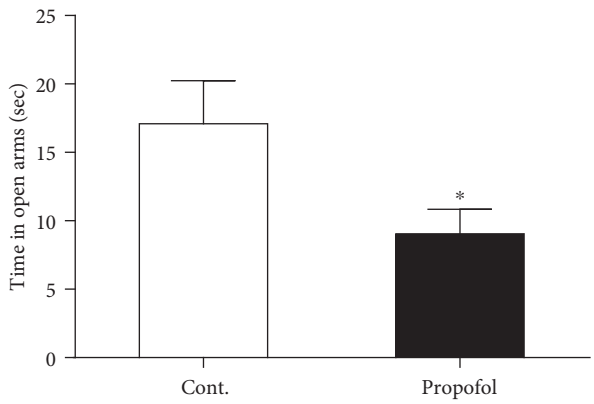

Figure 4: Propofol decreased the total time in the open arms of mice compared to control mice in the elevated plus maze test. All data are expressed as the means ± SEM. $n = 10$. $^{*}p < 0.05$.

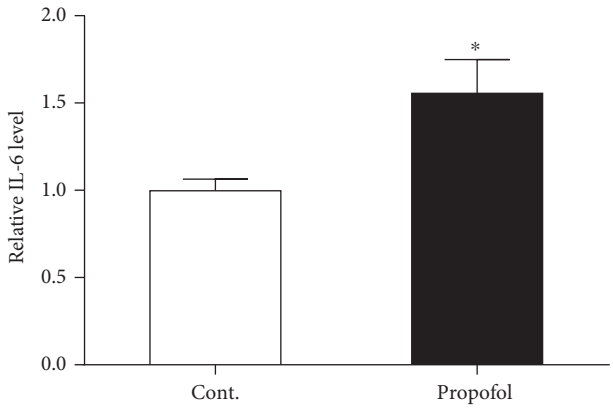

Figure 5: Propofol increased the IL-6 level in the brain tissues of mice compared to control mice. All data are expressed as the means ± SEM. $n = 6$. $^{*}p < 0.05$.

microglial cell was activated in the brain of mice after propofol exposure. The microglial cell protein marker, CD11b that is one of the common marker proteins to demonstrate the activation of microglia, was investigated with western blot. Surprisingly, our results indicated that propofol could inhibit the protein level of CD11b. The plausible explanation here is propofol could inhibit the CD11b protein expression regardless of whether the microglial cells were activated or not. The prominent advancement of propofol may be important for the potential treatment of alleviating the microglial overactivation in some pathological conditions of the CNS, such as trauma, stroke, and multiple sclerosis. We also looked into the effect of propofol on the synaptic proteins that are very important fundamental factors behind the behavioural performances of animals [15]. Interestingly, although propofol could affect the microglial cell function, it seemed not to impact the synaptic protein expression level. These findings are not in line with some previous reports [16]. The possible reason may be due to the difference of time window when the proteins were collected after the propofol was given. But our findings still suggested that propofol might have a different impact on neuron and other glial cells. In the future study, the effects of propofol on glial and neuronal cells should be elaborated individually.

The neuroinflammatory response in the CNS exposed to the single dose of propofol was further validated by the ELISA test of IL-6 in these two groups of mice. We found propofol treatment could induce an upregulation of IL-6 in the mouse brain. These findings were in line with the results of propofol on the microglial cell function. While inhibiting the CD11b component, propofol may activate the relevant signaling pathways that are important for the microglial cell activation.

Collectively, our study suggested propofol was able to influence the microglial cell function and exacerbate inflammatory response in the CNS. The cellular and molecular reaction might be responsible for the behavioural changes in these mice that are administrated with the single dose of propofol. Our study emphasized the important role of anaesthetics on the impact of mood changes after operation, but mood could be significantly impacted by many factors, including inflammation [17] that is quite often seen in the

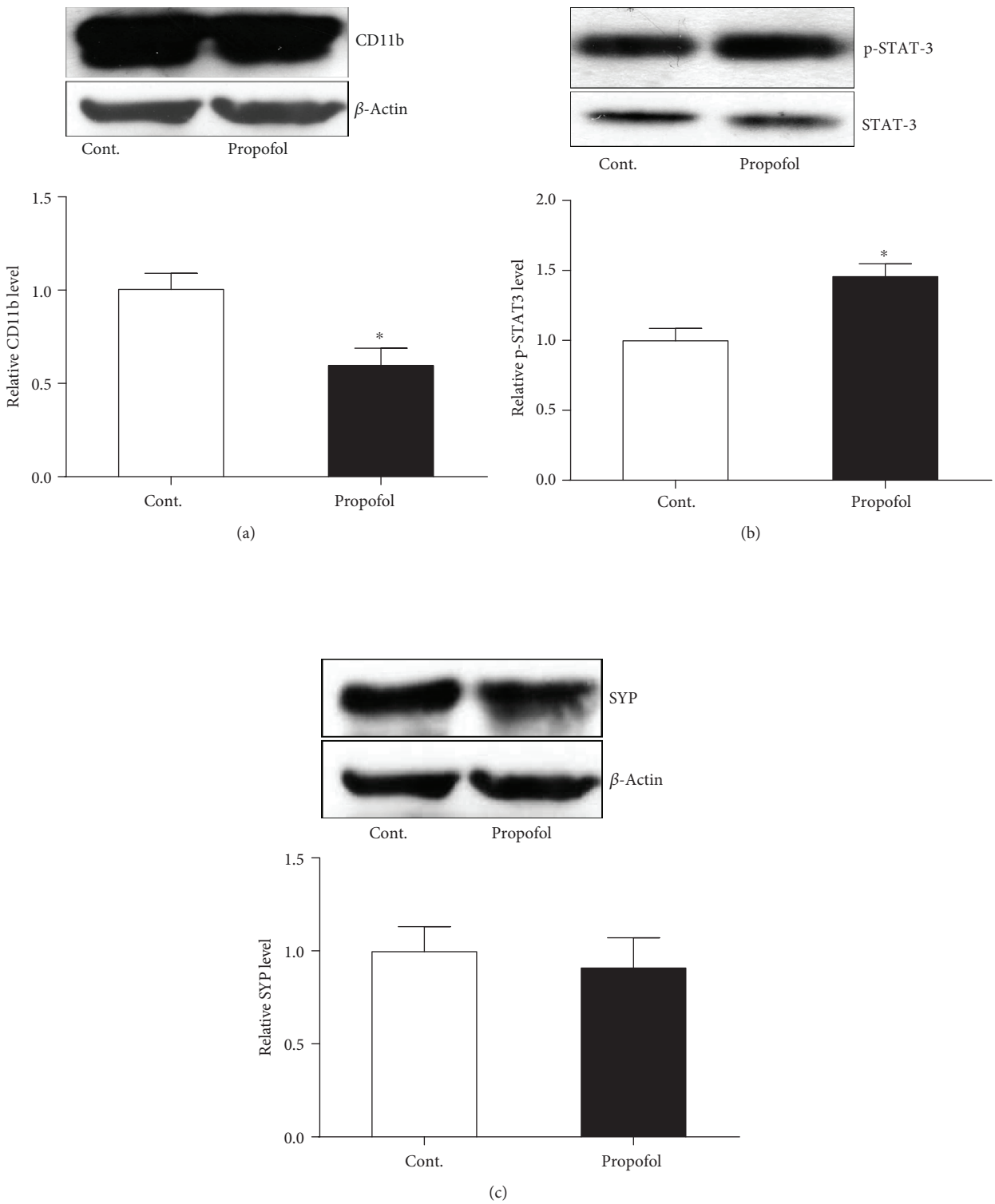

Figure 6: Propofol influenced the function of astrocytes and STAT-3 in the brain tissues of mice compared to control mice. (a) Propofol decreased the expression of the CD11b protein in the brain of mice. (b) Propofol increased the expression of the p-STAT-3 protein in the brain of mice. (c) Propofol did not change the expression of the SYP protein in the brain of mice. All data are expressed as the means ± SEM. $n = 5$. $^{*}p < 0.05$.

patients during and after surgery. Therefore, a series of well-designed, systemic studies on an animal model which involved not only the anaesthetics but also the surgery procedure are warranted for the future.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All the authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] M. M. Ghoneim and M. W. O'Hara, "Depression and postoperative complications: an overview," *BMC Surgery*, vol. 16, no. 1, p. 5, 2016.

[2] A. Burns, S. Banerjee, J. Morris et al., "Treatment and prevention of depression after surgery for hip fracture in older people: randomized, controlled trials," *Journal of the American Geriatrics Society*, vol. 55, no. 1, pp. 75–80, 2007.

[3] S. Y. Kim, S. W. Kim, I. S. Shin et al., "Changes in depression status during the year after breast cancer surgery and impact on quality of life and functioning," *General Hospital Psychiatry*, vol. 50, pp. 33–37, 2018.

[4] M. S. Walker, D. M. Zona, and E. B. Fisher, "Depressive symptoms after lung cancer surgery: their relation to coping style and social support," *Psychooncology*, vol. 15, no. 8, pp. 684–693, 2006.

[5] L. Kayaalp, P. Bozkurt, G. Odabasi et al., "Psychological effects of repeated general anesthesia in children," *Paediatric Anaesthesia*, vol. 16, no. 8, pp. 822–827, 2006.

[6] J. Barr, "Propofol: a new drug for sedation in the intensive care unit," *International Anesthesiology Clinics*, vol. 33, no. 1, pp. 131–154, 1995.

[7] U. Goswami, S. Babbar, and S. Tiwari, "Comparative evaluation of the effects of propofol and sevoflurane on cognitive function and memory in patients undergoing laparoscopic cholecystectomy: a randomised prospective study," *Indian Journal of Anaesthesia*, vol. 59, no. 3, pp. 150–155, 2015.

[8] H. C. Alves, A. M. Valentim, I. A. S. Olsson, and L. M. Antunes, "Intraperitoneal anaesthesia with propofol, medetomidine and fentanyl in mice," *Laboratory Animals*, vol. 43, no. 1, pp. 27–33, 2009.

[9] J. Wang, Y. Zhang, H. Xu et al., "Fluoxetine improves behavioral performance by suppressing the production of soluble β-amyloid in APP/PS1 mice," *Current Alzheimer Research*, vol. 11, no. 7, pp. 672–680, 2014.

[10] Y. S. Mineur, C. Belzung, and W. E. Crusio, "Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice," *Behavioural Brain Research*, vol. 175, no. 1, pp. 43–50, 2006.

[11] D. J. David, B. A. Samuels, Q. Rainer et al., "Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression," *Neuron*, vol. 62, no. 4, pp. 479–493, 2009.

[12] J. Wang, S. Zhu, H. Wang et al., "Astrocyte-dependent protective effect of quetiapine on GABAergic neuron is associated with the prevention of anxiety-like behaviors in aging mice after long-term treatment," *Journal of Neurochemistry*, vol. 130, no. 6, pp. 780–789, 2014.

[13] T. Kawano, D. Yamanaka, B. Aoyama et al., "Involvement of acute neuroinflammation in postoperative delirium-like cognitive deficits in rats," *Journal of Anesthesia*, vol. 32, no. 4, pp. 506–517, 2018.

[14] S. W. Jeon and Y. K. Kim, "The role of neuroinflammation and neurovascular dysfunction in major depressive disorder," *Journal of Inflammation Research*, vol. Volume 11, pp. 179–192, 2018.

[15] R. S. Duman and G. K. Aghajanian, "Synaptic dysfunction in depression: potential therapeutic targets," *Science*, vol. 338, no. 6103, pp. 68–72, 2012.

[16] K. Y. Lee, Y. Jang, M. H. Lee et al., "Intravenous anesthetic, propofol affects synaptic responses in cerebellar Purkinje cells," *Clinical Psychopharmacology and Neuroscience*, vol. 16, no. 2, pp. 176–183, 2018.

[17] J. C. Felger, "Imaging the role of inflammation in mood and anxiety-related disorders," *Current Neuropharmacology*, vol. 16, no. 5, pp. 533–558, 2018.

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Research Article

Irisin Attenuates Neuroinflammation and Prevents the Memory and Cognitive Deterioration in Streptozotocin-Induced Diabetic Mice

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Diabetes mellitus (DM) patients experience memory and cognitive deficits. The mechanisms underlying this dysfunction in the brain of DM patients are not fully understood, and therefore, no optimized therapeutic strategy has been established so far. The aim of the present study was to assess whether irisin was able to improve memory and cognitive performance in a streptozotocin-induced diabetic mouse model. A diabetic mouse model was established and behavioral tests were performed. We also set up primary cultures for mechanism studies. Western blots and EMSA were used for molecular studies. Significant impairment of cognition and memory was observed in these DM mice, which could be effectively prevented by irisin cotreatment. We also found upregulated levels of GFAP protein, reduced synaptic protein expression, and increased levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the brains; however, irisin significantly attenuated these cellular responses. Meanwhile, our results demonstrated that irisin inhibited the activation of P38, STAT3, and NFκB proteins of DM mice. Furthermore, our results suggested that irisin might regulate the function of P38, STAT3, and NFκB in hippocampal tissues of DM mice. Collectively, irisin inhibited neuroinflammation in STZ-induced DM mice by inhibiting cytokine release and improving their cognitive function. Our findings revealed the mechanism of irisin's anti-inflammatory effect in the CNS.

1. Introduction

Diabetes is a chronic metabolic disorder and is becoming a global public health problem. It results in a significant increase of morbidity and mortality, placing heavy economic burdens on families and health care systems [1]. There is strong evidence that DM increases the risk of cognitive and memory deterioration [2]. Both diabetes and cognitive and memory impairment occur more commonly in elderly people. In DM, one of the prominent pathological changes is hyperglycemia that results from increased glucose production in the liver but decreased insulin production in the pancreas [3]. Such deficits likely contribute to the memory and cognitive dysfunction in DM. Clinical studies have also revealed that many pediatric and young adult patients with DM manifested lower attention and compromised executive functions [4]. Moreover, poor glycemic control in DM accelerated the rate of cognitive decline over time, while improving metabolic control could ameliorate the decline [5]. Meanwhile, around 70%-80% of Alzheimer's disease (AD) patients may have diabetes or abnormal glucose metabolism [6]. The rapid deterioration of cognition in diabetic patients compared to that of nondiabetic patients has been observed in a previous report [7]. These findings especially implied that the consequences of the changes in the central nervous system (CNS) of DM, such as memory and cognition

dysfunction, were significantly due to the long-term glucose abnormality. Therefore, it is crucial to decipher the mechanism and develop new strategies to reduce the harm.

Irisin, also known as FNDC5, is a myokine that can increase energy expenditure by accelerating the "browning" of white adipose tissue [8]. Irisin is cleaved from its precursor fibronectin type III domain-containing protein 5 and is associated with better glucose homeostasis by attenuating insulin resistance [8]. Results from some studies have suggested that irisin could be a treatment option for obesity and associated diseases such as type 2 DM [9]. More importantly, recent studies demonstrated that irisin treatment improved endothelial dysfunction in diet-induced diabetic mice [10]. Endothelial dysfunction is a possible underlying mechanism by which memory and cognition deterioration is precipitated in DM animals [11]. Therefore, irisin is a promising candidate to prevent memory and cognition deterioration in diabetic patients.

Astrocytes, the most abundant cell in the CNS, support neighboring neurons and constitute the tripartite synapses [12]. Accumulating evidences have implied that astrocytes play an important role in memory and cognitive function [12]. A recent report demonstrated that repairing damaged astrocytes could improve the cognitive impairments of STZ-diabetic mice [13]. Therefore, it is reasonable to postulate that irisin exerts beneficial effects on DM mice by acting on astrocytes.

The aim of the present study is to assess (a) whether irisin can exert beneficial effects on the neuropathological changes in DM mice, specifically for astrocyte activation, neuroinflammation, and synaptic protein loss; (b) whether the astrocyte is one of the therapeutic targets of irisin; (c) whether irisin is capable of improving memory and cognitive performance and protecting neurons against astrocyte-mediated neuronal damage; and (d) whether irisin can regulate the signaling pathways which trigger the neuroinflammation cascade.

2. Materials and Methods

2.1. Animals and Drugs. We used 8-week-old male C57BL/6J mice for this animal study. All mice were on a 12 h light : 12 h dark cycle with free access to food and water. Mice were treated according to the guidelines established by the Chinese Council on Animal Care and all procedures were approved by the Animal Care Committee of Qingdao Municipal Hospital, China and Qilu Hospital of Shandong University, China.

Mice were randomly grouped into 4 groups: control, control plus irisin (0.5 mg/kg/day), STZ (150 mg/kg), and STZ plus irisin (0.5 mg/kg/day). Both STZ and irisin were bought from Sigma-Aldrich (MO, USA). STZ was dissolved in distilled 0.1 mmol/l sodium citrate buffer (pH 4.5) and experimental dosages of irisin were prepared in normal saline. We administered a single dose of STZ intraperitoneally to establish a diabetic mouse model. Meanwhile, irisin was given to mice through daily intraperitoneal injection as well. Behavioral tests were performed 3 weeks later.

2.2. Y Maze Test. Spatial working memory was assessed by the Y maze test as previously described [14]. The experimental apparatus consisted of 3 arms (35 cm long, 25 cm high, and 10 cm wide, labeled A, B, or C) diverging at a 120° from the central point. The apparatus was placed 40 cm above the floor in a room with dim light. We individually placed mice at the end of the start arm and allowed them to move freely through the maze during an 8-minute session. A mouse was considered to have entered an arm when all 4 paws were positioned in the arm runway. The sequence of arm entries and the total number of entries over a period of 8 min were recorded. The percentage of alternation was defined as the number of sequential triplets containing entries into all three arms (A-B-C and A-C-B constituted a sequential triplet, while A-C-A or A-B-A did not) during the session as a proportion of the maximum possible alternation (equivalent to the total number of arm entries minus -2) $\times 100$ [14]. Mice whose total entrance number is less than 15 times during the test were not taken into the final data.

2.3. Novel Object Recognition. Nonspatial memory was measured using the novel object recognition (NOR) test as previously described [15]. The test was conducted in a Plexiglas activity box (40 cm × 40 cm × 22 cm). Mice were given 15 min daily to explore the empty box for 3 consecutive days prior to the behavioral test. On the fourth day, mice were presented with two identical objects (A1 and A2) for a period of 10 min. After 1 hr, mice were put back into the box and exposed to two objects for 5 min, in which one of the familiar objects (A2) was replaced by a novel object B. Animal activity was considered object exploration if the mice's nose touched the object or if the mice were facing the object within a distance of around 2 cm from the object [15].

2.4. Collection of Cerebrospinal Fluid (CSF) Samples. CSF was collected from the cisterna magna, as previously reported with minor modifications [12]. After anesthesia with isoflurane, we made a sagittal incision on the inferior line of the occiput. Then, we separated the subcutaneous tissue and muscles in the surrounding area to clearly expose the meninges. We could collect about 3-4 μl of CSF in the glass tube after penetrating a capillary tube into the translucent meninges.

2.5. Western Blot Analysis. Protein samples were boiled and run on SDS-PAGE gels, followed by electrophoretically transferring onto nitrocellulose membranes. The nitrocellulose membranes were then blocked with 5% nonfat dried milk in TBST buffer. The blocked membranes were incubated with an antibody to glial fibrillary acidic protein (GFAP) (1 : 4000; Millipore Corp., MA, USA), synaptophysin (SYP) (1 : 4000, Abcam, Cambridge, United Kingdom), p38 MAPK (1 : 1000; New England Biolabs, Beverly, MA), phospho-p38 MAPK (1 : 1000; New England Biolabs, Beverly, MA), and p-Stat3 (1 : 1000; Cell Signaling Technology, Danvers, MA) and Stat 3 (1 : 1000; Cell Signaling Technology, Danvers, MA) in TBST milk overnight at 4°C. We used β-actin as a loading control (1 : 5000; Santa Cruz Biotechnology Inc., CA, USA). Values used for statistical analysis were

expressed as the ratio of the band of each protein to the band of their loading control.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). We measured the concentrations of IL-6 and IL-1β in the present study with an ELISA kit (eBioscience, Thermo Fisher Scientific). Each sample was loaded in a duplicate manner with appropriate dilutions to make sure their luminescent units fell within the linear range of standard curves. The values were normalized and expressed as the ratio of each sample to their total loading protein.

2.7. Electrophoretic Mobility Shift Assay (EMSA). EMSA was carried out according to a previous report with a minor modification [16]. Nuclear proteins were extracted from mouse brains. The protein of each sample was probed with excess ^{32}P-end-labeled oligonucleotides with a consensus sequence specific for NFκB/DNA (Promega Corp., Madison, WI). The mixture was then fractionated on a 6% polyacrylamide gel for around 2 hours (180 V). The gel was then placed on filter paper and dried. After that, the gel was exposed to a film at 270°C for 1.5 hours followed by autoradiography.

2.8. Statistical Analysis. Data in the present study was expressed as the mean ± SEM. The data was then analyzed with one-way ANOVA, followed by the Newman-Keuls *post hoc* test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Irisin Improved the Memory and Cognitive Deficiency in Diabetic Mice. We tested whether DM mice could demonstrate memory and cognition deficiency in the Y maze and NOR tests. First, short-term spatial memory was investigated with the Y maze. As shown in Figure 1, DM mice showed a significant decrease of alternation behavior compared to the control mice (Figure 1(a)), while the total travel distances were comparable between these two groups (Figure 1(b)). As shown in Figure 1(a), cotreatment with irisin could significantly improve the reduced alternation in DM mice without affecting the total travel distances (Figure 1(b)). These results in the Y maze suggested that short-term spatial memory and spontaneous alternation were significantly reduced in the STZ-induced DM mouse model. Next, nonspatial visual discrimination memory was measured with the NOR test. The NOR test measures the ability of mice to tell between a novel object and a familiar object. To compare the performance of mice in different groups, the time spent interacting with objects (novel or familiar) was measured and a discrimination ratio was used (the ratio of time spent exploring the novel object to the time spent exploring both objects). Mice treated with STZ exhibited a low discrimination ratio (Figure 2(a)). Cotreatment with irisin prevented the STZ-induced memory deficits, while irisin alone did not affect the memory performance of mice (Figure 2(a)). The difference in the behavioral performance was not attributed to alterations in locomotion as the total distance traveled and the total time spent interacting with the objects did not show a significant difference between these groups (Figure 2(b)).

3.2. Irisin Inhibited the Increase of GFAP and Prevents Synaptic Protein Loss in Diabetic Mice. Astrocyte activation is closely related with cognitive dysfunction [12, 17]. We tested with western blot whether the astrocyte activation marker, GFAP, was regulated in these DM mice. As shown in Figure 3(a), STZ treatment induced a significant increase of GFAP protein expression, while irisin could prevent the upregulated protein level. A previous study also suggested that the loss of the presynaptic vesicle protein synaptophysin (SYP) in the hippocampus correlates with cognitive decline in human patients [18]. To understand why irisin improved the behavior of DM mice, brain tissue was processed and measured with western blot to investigate whether irisin could prevent synaptic protein loss in diabetic mice. We found that STZ caused the reduced expression of SYP in DM mice, which could be effectively attenuated by irisin cotreatment (Figure 3(b))

3.3. Irisin Attenuated the IL-1β and IL-6 Levels in Hippocampal Tissues and CSF of Diabetic Mice. Since we found that irisin could inhibit astrocyte activation in DM mice and improve cognition and memory, we postulated whether neuroinflammation was a response to the neuronal deficiency in these DM mice. Firstly, we tested the expression of IL-1β and IL-6 in brain hippocampal tissues of all groups. Our results indicated that STZ caused an obvious increase in IL-1β and IL-6 levels in DM, which could be prevented by irisin treatment (Figure 4). Secondly, we tested the level of these two cytokines in the CSF of these mice. We found consistent results of their expression level in CSF (Figure 5). The above results suggested that irisin might attenuate the neuroinflammation in DM mice by reducing the levels of IL-1β and IL-6 in their brains.

3.4. Irisin Reduced the Activation of P38, STAT3, and NFκB Proteins in the Brain Hippocampal Tissues of Diabetic Mice. We also explored the possible underlying mechanism that was responsible for the neuropathological changes in DM mice. Phosphorylation of P38 and STAT3 is closely involved in the cytokine cascade [19, 20]. Therefore, we tested whether the activation of P38 and STAT3 was involved in the response of irisin treatment in these DM mice. The phosphorylation status of P38 and STAT3 was investigated with western blot. We found that STZ caused the increased phosphorylated protein level of P38 in the hippocampal tissues, which could be reduced by cotreatment of irisin (Figure 6(a)). The phosphorylation of STAT3 was measured in the following western blot study. As shown in Figure 6(b), STZ alone induced the upregulated expression level of phosphorylated STAT3 but cotreatment prevented the upregulation. We measured the NFκB activation with EMSA. We found that the binding activity of NFκB to DNA was significantly increased in the brain tissue of DM mice (Figure 6(c)), indicating that NFκB was activated. Treatment with irisin effectively blocked the activation of the NFκB/DNA binding activity in these mice.

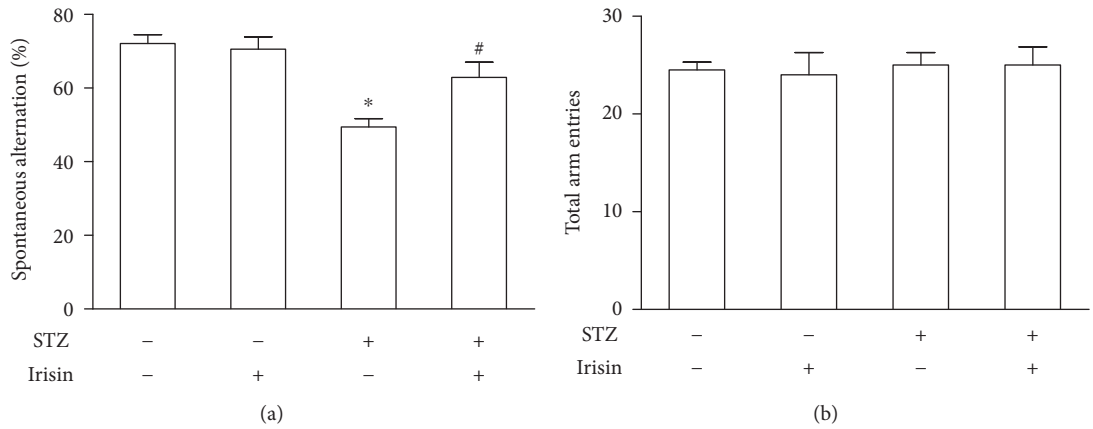

Figure 1: Irisin prevented STZ-induced working memory deterioration in DM mice. (a) STZ treatment caused significantly decreased spontaneous alternation of DM mice in the Y maze test. Irisin could prevent the decrease of the working memory performance of DM mice. (b) Neither STZ nor irisin could change the total arm entries of these mice. All data are expressed as means ± SEM. $n = 10$. $^{*}p < 0.05$ vs. STZ (-) and irisin (-); $^{\#}p < 0.05$ vs. STZ (+) and irisin (-).

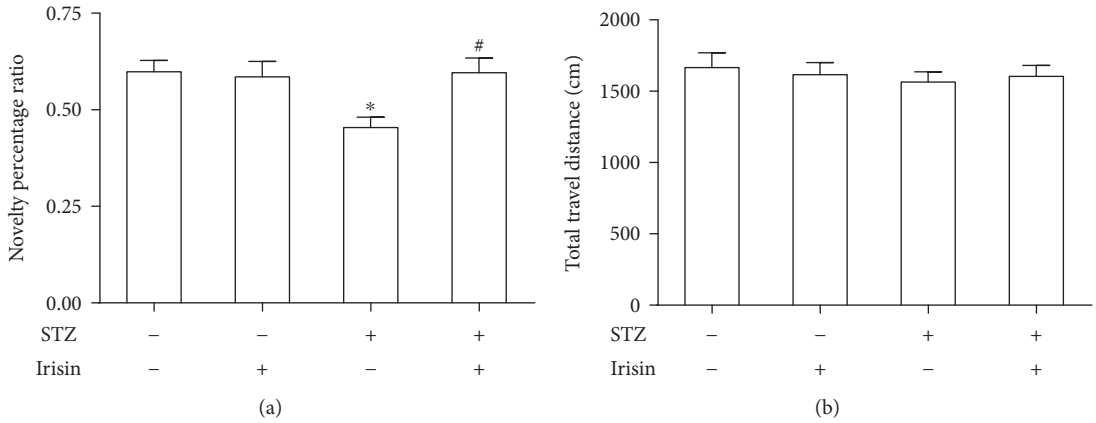

Figure 2: Irisin attenuated memory loss in STZ-induced DM mice. (a) Statistical analysis of the effect of irisin on memory deficits in DM mice. (b) There is no statistical difference of total travel distance during the test among these groups. All data are expressed as means ± SEM. $n = 10$. $^{*}p < 0.05$ vs. STZ (-) and irisin (-); $^{\#}p < 0.05$ vs. STZ (+) and irisin (-).

4. Discussion

Diabetes has detrimental effects on cognitive functioning. These effects are particularly prominent for memory function [21]. However, the underlying mechanisms by which DM affects the CNS remain largely unknown. And therefore, the current therapeutic strategies are far from satisfactory due to the lack of specific medications.

In this study, we explored the possible application of irisin in the treatment of memory and cognitive dysfunction in DM. Diabetic mice were established by treating with STZ, and the effects of irisin on behavioral performances were measured with Y maze and NOR tests. For the first time, our data suggested that irisin could significantly improve the memory and cognitive function in DM mice (Figures 1 and 2). Although irisin is not an approved compound for the treatment of DM, a recent study has provided evidence that irisin could exert therapeutic potential in obesity and type 2 DM by stimulating the "browning" of white adipose tissue [22]. Our findings are consistent with the hypothesis that irisin might be a good candidate for the future treatment of DM since our results here also provided new evidences supporting the therapeutic benefits of irisin by improving memory and cognition in the DM mouse model.

In the CNS, especially in the hippocampus of diabetic mice, an enhanced inflammatory response with astrocyte activation has been observed, which was possibly responsible for the consequent neuronal deterioration [23]. Astrocyte activation was observed in a high-fat feeding animal model [24]. Consistently, we found a significantly increased expression of the astrocyte activation marker protein, GFAP, along with a significantly decreased level of the presynaptic protein, SYP (Figure 3). These results suggested astrocyte activation and synaptic dysfunction in these DM mice. The assumption was further partially supported by our ELISA data with the upregulated expression of two cytokines, IL-1β and IL-6, in brain hippocampal tissues and CSF. Interestingly, the above changes in DM mice could be effectively prevented by cotreatment with irisin.

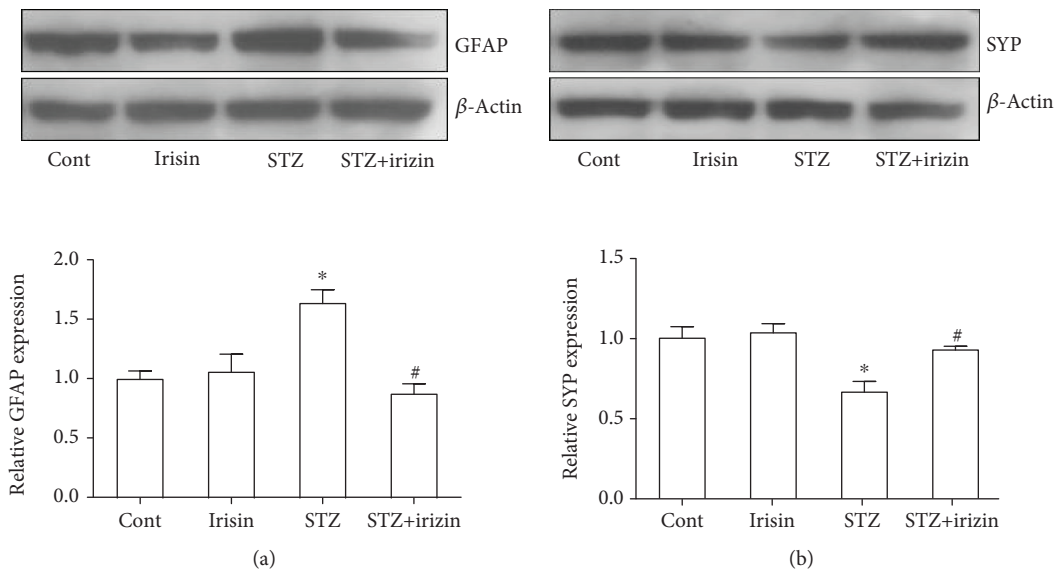

Figure 3: Irisin attenuates the abnormal expression level of GFAP and SYP in DM mouse exposed to STZ. (a) Representative blot picture showing the expression of the GFAP protein in DM mice and the statistical bar graph showing the results. (b) Representative blot picture showing the expression level of SYP in DM mice and the statistical bar graph showing the results. All data are expressed as means ± SEM. $n = 5$. $^{*}p < 0.05$ vs. Cont; $^{\#}p < 0.05$ vs. STZ.

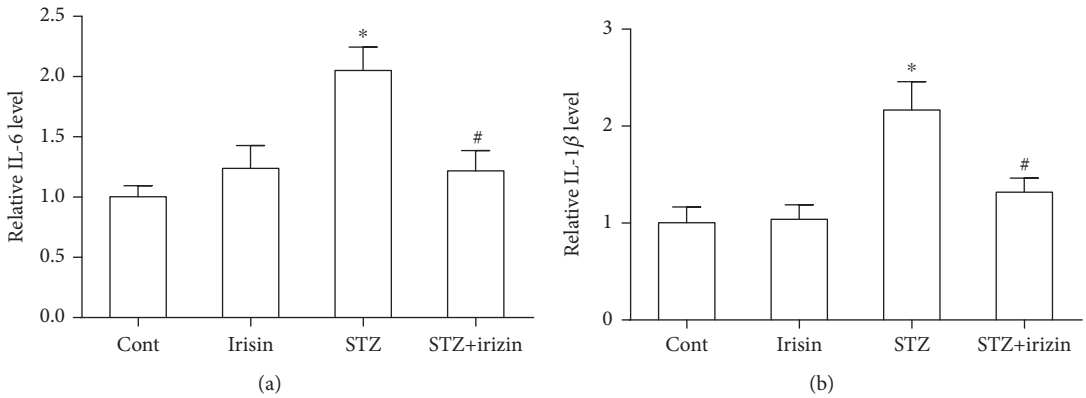

Figure 4: Irisin ameliorated the increased level of IL-6 and IL-1β in the hippocampal tissues of DM mice exposed to STZ. (a) Statistical bar graph showing irisin reduced the STZ-induced increased expression level of IL-6 in hippocampal tissues of DM mice. (b) Statistical bar graph showing irisin reduced the STZ-induced increased expression level of IL-1β in hippocampal tissues of DM mice. All data are expressed as means ± SEM. $n = 6$. $^{*}p < 0.05$ vs. Cont; $^{\#}p < 0.05$ vs. STZ.

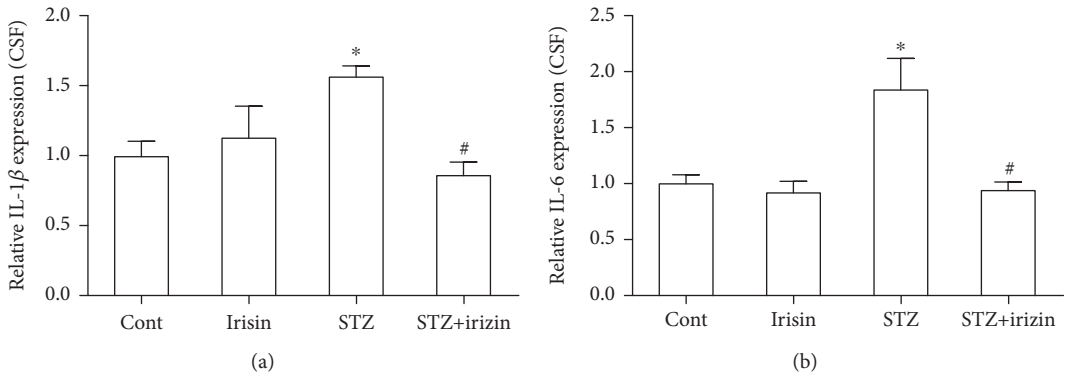

Figure 5: Irisin reduced the level of IL-6 and IL-1β in the CSF of DM mice exposed to STZ. (a) STZ increased the level of IL-1β in the CSF of DM mice, but irisin treatment could reduce the upregulation of IL-1β. (b) STZ increased the level of IL-6 in the CSF of DM mice, and irisin treatment could reduce the upregulation of IL-6. All data are expressed as means ± SEM. $n = 6$. $^{*}p < 0.05$ vs. Cont; $^{\#}p < 0.05$ vs. STZ.

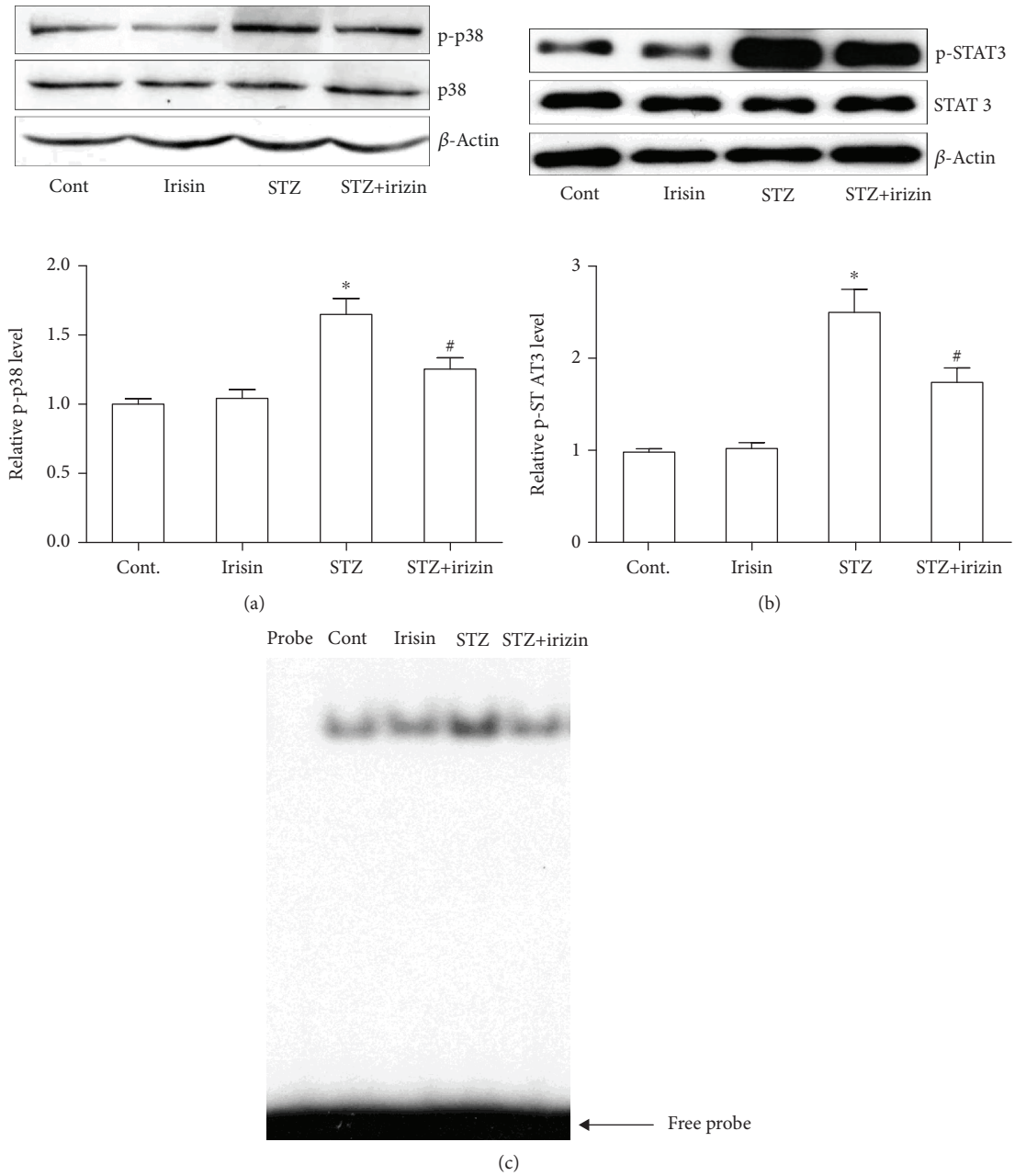

Figure 6: Irisin reduced the activation of p38, STAT3, and NFκB proteins in the brain hippocampal tissues of DM mice. (a) Representative western blot photograph and statistical results of p-p38 and total p38 in hippocampal tissues of all groups. (b) Representative western blot photograph and statistical results of p-STAT3 and total STAT3 in hippocampal tissues of all groups. (c) NFκB/DNA binding activity was determined by EMSA. All data are expressed as means ± SEM. $n = 5$. $^{*}p < 0.05$ vs. Cont; $^{\#}p < 0.05$ vs. STZ.

Previous studies have well established the role of p38 and STAT3 in neuroinflammation [25, 26]. In the present study, notable changes of the phosphorylation level of p38 and STAT3 were shown in a western blot study (Figure 6). STAT3 is one of key factors involved in many cytokine cascades, including IL-6, IL-10, and TNFα [27]. And a recent study disclosed that the JAK/STAT and STAT3 pathway played a key role in the induction of NMDA-receptor-dependent long-term depression in the hippocampus [28]. Moreover, the STAT3 in astrocytes is an essential step by which astrocytes play a proinflammatory response in the CNS [29]. Our findings here further highlighted the importance of STAT3 in memory and cognitive function, especially in the pathological setting, such as in elderly patients with DM.

To the best of our knowledge, the present study provides the first evidence that irisin prevents memory and cognitive deficits via regulating JAK/STAT and STAT3 and consequent inflammatory injury in the brains of DM mice. We anticipate that our findings here will provide the foundation

for future clinical trials to determine whether irisin may prevent or treat memory and cognitive dysfunction in DM patients while attenuating glucose abnormality.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare that there are no conflicts of interest in this study.

Acknowledgments

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References

[1] K. M. V. Narayan, E. W. Gregg, A. Fagot-Campagna, M. M. Engelgau, and F. Vinicor, "Diabetes — a common, growing, serious, costly, and potentially preventable public health problem," *Diabetes Research and Clinical Practice*, vol. 50, Supplement 2, pp. S77–S84, 2000.

[2] E. Saedi, M. R. Gheini, F. Faiz, and M. A. Arami, "Diabetes mellitus and cognitive impairments," *World Journal of Diabetes*, vol. 7, no. 17, pp. 412–422, 2016.

[3] D. A. Butterfield, F. Di Domenico, and E. Barone, "Elevated risk of type 2 diabetes for development of Alzheimer disease: a key role for oxidative stress in brain," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1842, no. 9, pp. 1693–1706, 2014.

[4] C. M. Ryan, E. van Duinkerken, and C. Rosano, "Neurocognitive consequences of diabetes," *American Psychologist*, vol. 71, no. 7, pp. 563–576, 2016.

[5] O. Ojo and J. Brooke, "Evaluating the association between diabetes, cognitive decline and dementia," *International Journal of Environmental Research and Public Health*, vol. 12, no. 7, pp. 8281–8294, 2015.

[6] J. Janson, T. Laedtke, J. E. Parisi, P. O'Brien, R. C. Petersen, and P. C. Butler, "Increased risk of type 2 diabetes in Alzheimer disease," *Diabetes*, vol. 53, no. 2, pp. 474–481, 2004.

[7] R. Ravona-Springer, X. Luo, J. Schmeidler et al., "Diabetes is associated with increased rate of cognitive decline in questionably demented elderly," *Dementia and Geriatric Cognitive Disorders*, vol. 29, no. 1, pp. 68–74, 2010.

[8] N. Perakakis, G. A. Triantafyllou, J. M. Fernández-Real et al., "Physiology and role of irisin in glucose homeostasis," *Nature Reviews Endocrinology*, vol. 13, no. 6, pp. 324–337, 2017.

[9] T. Hofmann, U. Elbelt, and A. Stengel, "Irisin as a muscle-derived hormone stimulating thermogenesis – A critical update," *Peptides*, vol. 54, pp. 89–100, 2014.

[10] N. Hou, G. Du, F. Han, J. Zhang, X. Jiao, and X. Sun, "Irisin Regulates Heme Oxygenase-1/Adiponectin Axis in Perivascular Adipose Tissue and Improves Endothelial Dysfunction in Diet-Induced Obese Mice," *Cellular Physiology and Biochemistry*, vol. 42, no. 2, pp. 603–614, 2017.

[11] B. Sharma and N. Singh, "Pitavastatin and 4-Hydroxy-3-Methoxyacetophenone (HMAP) Reduce Cognitive Dysfunction in Vascular Dementia During Experimental Diabetes," *Current Neurovascular Research*, vol. 7, no. 3, pp. 180–191, 2010.

[12] J. Qiao, J. Wang, H. Wang et al., "Regulation of astrocyte pathology by fluoxetine prevents the deterioration of Alzheimer phenotypes in an APP/PS1 mouse model," *Glia*, vol. 64, no. 2, pp. 240–254, 2016.

[13] M. Nakano, K. Nagaishi, N. Konari et al., "Bone marrow-derived mesenchymal stem cells improve diabetes-induced cognitive impairment by exosome transfer into damaged neurons and astrocytes," *Scientific Reports*, vol. 6, no. 1, 2016.

[14] J. Wang, S. Zhu, H. Wang et al., "Astrocyte-dependent protective effect of quetiapine on GABAergic neuron is associated with the prevention of anxiety-like behaviors in aging mice after long-term treatment," *Journal of Neurochemistry*, vol. 130, no. 6, pp. 780–789, 2014.

[15] J. Wang, Y. Zhang, H. Xu et al., "Fluoxetine improves behavioral performance by suppressing the production of soluble β-amyloid in APP/PS1 mice," *Current Alzheimer Research*, vol. 11, no. 7, pp. 672–680, 2014.

[16] H. Y. Hsiao, O. T. Mak, C. S. Yang, Y. P. Liu, K. M. Fang, and S. F. Tzeng, "TNF-α/IFN-γ-induced iNOS expression increased by prostaglandin E2 in rat primary astrocytes via EP2-evoked cAMP/PKA and intracellular calcium signaling," *Glia*, vol. 55, no. 2, pp. 214–223, 2007.

[17] G. Dallérac and N. Rouach, "Astrocytes as new targets to improve cognitive functions," *Progress in Neurobiology*, vol. 144, pp. 48–67, 2016.

[18] C.-I. Sze, J. C. Troncoso, C. Kawas, P. Moution, D. L. Price, and L. J. Martin, "Loss of the Presynaptic Vesicle Protein Synaptophysin in Hippocampus Correlates with Cognitive Decline in Alzheimer Disease," *Journal of Neuropathology and Experimental Neurology*, vol. 56, no. 8, pp. 933–944, 1997.

[19] Y. Wang, A. H. H. van Boxel-Dezaire, H. Cheon, J. Yang, and G. R. Stark, "STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor," *Proceedings of the National Academy of Sciences*, vol. 110, no. 42, pp. 16975–16980, 2013.

[20] D. C. Underwood, R. R. Osborn, C. J. Kotzer et al., "SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence," *Journal of Pharmacology and Experimental Therapeutics*, vol. 293, no. 1, pp. 281–288, 2000.

[21] M. Kumari, E. Brunner, and R. Fuhrer, "Minireview: mechanisms by which the metabolic syndrome and diabetes impair memory," *The Journals of Gerontology Series A*, vol. 55, no. 5, pp. B228–B232, 2000.

[22] P. Boström, J. Wu, M. P. Jedrychowski et al., "A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis," *Nature*, vol. 481, no. 7382, pp. 463–468, 2012.

[23] A. Nagayach, N. Patro, and I. Patro, "Astrocytic and microglial response in experimentally induced diabetic rat brain," *Metabolic Brain Disease*, vol. 29, no. 3, pp. 747–761, 2014.

[24] L. B. Buckman, M. M. Thompson, R. N. Lippert, T. S. Blackwell, F. E. Yull, and K. L. J. Ellacott, "Evidence for a novel functional role of astrocytes in the acute homeostatic response to high-fat diet intake in mice," *Molecular Metabolism*, vol. 4, no. 1, pp. 58–63, 2015.

[25] J. P. O'Callaghan, K. A. Kelly, R. L. VanGilder, M. V. Sofroniew, and D. B. Miller, "Early activation of STAT3 regulates reactive astrogliosis induced by diverse forms of neurotoxicity," *PLOS ONE*, vol. 9, no. 7, article e102003, 2014.

[26] X. Guo, C. Harada, K. Namekata et al., "Regulation of the severity of neuroinflammation and demyelination by TLR-ASK1-p38 pathway," *EMBO Molecular Medicine*, vol. 2, no. 12, pp. 504–515, 2010.

[27] C. F. Lai, J. Ripperger, K. K. Morella et al., "Receptors for interleukin (IL)-10 and IL-6-type cytokines use similar signaling mechanisms for inducing transcription through IL-6 response elements," *Journal of Biological Chemistry*, vol. 271, no. 24, pp. 13968–13975, 1996.

[28] C. S. Nicolas, S. Peineau, M. Amici et al., "The Jak/STAT pathway is involved in synaptic plasticity," *Neuron*, vol. 73, no. 2, pp. 374–390, 2012.

[29] Y. Lu, M. He, Y. Zhang et al., "Differential pro-inflammatory responses of astrocytes and microglia involve STAT3 activation in response to 1800 MHz radiofrequency fields," *PLOS ONE*, vol. 9, no. 10, article e108318, 2014.

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Review Article

Crosstalk between the Ketogenic Diet and Epilepsy: From the Perspective of Gut Microbiota

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Given the association between a range of neurological disorders and changes in the gut microbiota, interest in the gut microbiota has recently increased. In particular, the significant involvement of the autoimmune processes in the development of epilepsy, one of the most serious and widespread neurological diseases, has led to a suggested link with the gut microbiome. Because the constitution of the gut microbiome can be influenced by diet, dietary therapy has been shown to have a positive impact on a wide range of conditions via alteration of the gut microbiota. An example of one such diet is the ketogenic diet (KD), which promotes a diet that contains high levels of fat, adequate levels of protein, and low levels of carbohydrate. Due to the near-total elimination of carbohydrates from the individual's food in this ultra-high-fat diet, ketone bodies become an important source of energy. Although the ketogenic diet has proven successful in the treatment of refractory epilepsy and other illnesses, the underlying mechanisms of its neuroprotective effects have yet to be fully elucidated. Nevertheless, recent studies strongly indicate a role for the gut microbiota in the effective treatment of epilepsy with the ketogenic diet. The latest advances regarding the links between the ketogenic diet, gut microbiota, and epilepsy are reviewed in this article, with a particular focus on the role of the gut microbiota in the treatment outcome.

1. Introduction

The diverse population of microbes in the gastrointestinal tract, including archaea, bacteria, fungi, protozoa, and viruses, is generally referred to as the gut microbiota [1]. Investigation of the role of the gut microbiota in disease and health has recently attracted increasing interest, and a growing body of evidence suggests a role for the gut microbiota in a wide range of neurological disorders via gut-brain interactions [2–5].

Epilepsy is among the most serious and widespread neurological disorders and represents a major liability for the healthcare system [6]. Moreover, approximately one-third of epilepsy patients eventually develop drug resistance, defined as the failure to achieve lasting freedom from seizures after sufficient trials with two tolerated, correctly selected, and administered antiepileptic drugs, either as a single therapy or as part of a combined treatment regimen [7–9].

Significant involvement of autoimmune processes in the development of epilepsy has been noted in a growing body of research [10–12]. Because microbiota are also strongly correlated with autoimmunity [13, 14], it is plausible that the specific composition of the gut microbiota population could influence both the individual's susceptibility to epilepsy and the subsequent progression of the illness [15, 16].

A number of studies on epilepsy and microbiota have recently been published, and many have demonstrated the significant impact of diet on the composition of the gut microbiota and on the subsequent health of the individual [17, 18]. The high-fat, sufficient-protein, and very low-carbohydrate ketogenic diet (KD) was established early in the 1920s to reproduce the central metabolic impacts and associated antiseizure effects of fasting [19]. The KD has since found continued world-wide use in the treatment of drug-resistant epilepsy [20, 21]. The function of the gut microbiota in the treatment of epilepsy with the KD has

recently been examined, and some recent studies have indicated that the KD alters the gut microbiota of individuals in the diseased state [22]. In this review, the latest advances regarding the crosstalk among the ketogenic diet, the gut microbiota, and epilepsy are reviewed with a focus on the role of the gut microbiota.

2. Microbiota and Epilepsy

2.1. Microbiota-Brain Axis. The gut microbiota is a multifaceted ecological population that contains trillions of microorganisms that inhabit the animal digestive tract and exhibits a range of dynamic interactions with the host's immune system and assists in maintaining the metabolic dynamic equilibrium [23–25]. Rather than being a merely commensal relationship, the association between the host organism and the gut microbiota is a mutualistic symbiosis [26]. Interest in the function of the gut microbiota in brain disorders has recently increased, with studies indicating that alterations in the gut microbiota could influence the brain's behavioural, cognitive, and physiological functions [27–30]. Although the precise mechanism of crosstalk between the brain and the gut microbiota has yet to be fully elucidated, the effect of the microbiota on the brain has become a hot topic within the field of neuroscience [27–29]. Broadly speaking, the gut microbiota influences the brain via a number of pathways, including the endocrine, immune, and metabolic systems, in addition to the neuroanatomical route between the gut and the brain provided by the nervous system [31–34]. The two-way communication link between the gut and brain is termed the gut-brain axis, whilst the interaction between the gut microbiota and the gut-brain axis is termed the gut microbiota-gut-brain axis or the gut microbiota-brain axis [35]. There has been significant focus on the function of microbes in the gut microbiota-brain axis because the gut microbiota can be deliberately altered, thereby providing an independent variable that can be therapeutically manipulated [36].

2.2. Gut Microbiota and Epilepsy

2.2.1. Epilepsy Correlates with Gut Microbiota through Autoimmunity. Epilepsy is a widespread serious and chronic neurological disorder that can severely delay development, reduce brain function, and lead to a significant mortality rate [37, 38]. Approximately one-third of epileptic individuals are resistant to drugs and thus extremely difficult to treat [39]. Although the aetiology of epilepsy has yet to be fully elucidated, factors at play include both hereditary risk and environmental influences [40]. The possible link between autoimmunity and the onset of epilepsy has attracted significant interest, and one population-level epidemiological study reported the frequent cooccurrence of epilepsy and certain autoimmune diseases [10]. Although epilepsy has a prevalence of 0.4% in a typical population, its prevalence among individuals with autoimmune conditions is as high as 17.5% [41]. Hence, a significant number of epilepsy cases are autoimmune-related, as emphasised by observational studies that suggested that seizures may be controlled via immunotherapy [42]. The role of specific autoimmune mechanisms and their related pathogenic autoantibodies in seizures has therefore been subjected to increased scrutiny [11, 43]. The possibility of autoimmune-related epilepsy must be carefully considered, particularly in drug-resistant and difficult-to-treat cases [44]. This is any form of epilepsy involving clinical symptoms suggestive of pathogenic involvement of the immune system in the onset of seizures or in the development of neuronal injury after the seizure [45]. This condition characteristically affects otherwise-healthy children and is typified by the sudden onset of one or more of the following: cognitive deterioration, encephalopathy, focal seizures, or other focal neurological defects [46–48]. Additional research is needed to provide an enhanced understanding of the pathogenic mechanisms involved, to identify the ideal immunotherapy and to estimate the prognostic impact of treatment.

The involvement of the gut microbiota in the pathogenicity of autoimmune conditions has been clearly demonstrated [49]. The gut microbiota can be both beneficial and harmful to the host [50]. They are essential for digestive processes and for maintaining homeostasis but are also involved in the development of autoimmune diseases through their function in controlling both the anti- and proinflammatory immune responses [51, 52]. Thus, the immune system and the gut microbiota are intimately linked and simultaneously affect each other [53]. Moreover, modulation of the immune system by the microbiota involves not only the intestinal environment but also the nervous system [54]. Hence, recent studies have examined the involvement of the peripheral and CNS-resident immune pathways in microbiota-gut-brain communication in healthy individuals and in those with neurological disorders [9, 54, 55].

Based on the crosstalk between autoimmunity, the onset of epilepsy, and microbiota, it is feasible that the constitution of the gut microbiota population could influence both the individual's susceptibility to epilepsy and the subsequent progression of the disease [15, 56]. Experimental research has indicated a significant link between the onset of epilepsy and elevated levels of proinflammatory cytokines such as IL-6 and IL-1β [57, 58], and it is well proven that cytokines are a key to driving and regulating human Th17 responses [59]. Hence, the onset of autoimmune-linked epilepsy could be triggered by commensal microbiota via spontaneous secretion of proinflammatory cytokines leading to an increase in Th17 cells [60].

2.2.2. Recent Studies of Epilepsy Involving the Gut Microbiota. Although investigations into the link between epilepsy and the intestinal microbiota remain in their infancy [61], Table 1 presents a number of studies with published results.

For instance, the 16s ribosomal DNA obtained from faecal samples was subjected to high-throughput sequencing to examine the microbiome compositions of 49 drug-sensitive epilepsy patients, 42 drug-resistant epilepsy patients, and a control group of 65 healthy individuals [62]. The gut microbial population of the drug-resistant individuals was found to be considerably altered relative to that of the control group, with anomalously raised levels of uncommon flora [62]. Moreover, elevated levels of *Lactobacillus* and *Bifidobacteria*

Table 1: Main findings of studies on gut microbiota and epilepsy.

Subjects	Age	Population	Methodology	Findings	Year	Authors
Human	5-50 years old	Drug-resistant epilepsy ($n = 42$), drug-sensitive epilepsy ($n = 49$), and healthy control ($n = 65$).	16s rRNA-based metagenomics	An abnormally increased abundance of rare flora. Bifidobacteria↑ and Lactobacillus↑ in patients with fewer seizures (no more than 4 seizures per year).	2018	Peng *et al.* [62]
Sprague-Dawley rats	45 days old	Chronic-stressed rats and sham-stressed rats.	Faecal microbiota transplantation (FMT) to recipients, in which commensal microbiota had been depleted by antibiotics	Perturbations in the gut microbiome, particularly those associated with chronic stress, in those with increased susceptibility to epilepsy.	2018	Medel-Matus *et al.* [63]
Human	22 years old	A girl with Crohn's disease (CD) and a 17-year history of epilepsy.	Faecal microbiota transplantation (FMT)	FMT achieved remission of intestinal and neurological symptoms in a girl with CD and a 17-year history of epilepsy. The finding inspires a novel treatment for epilepsy through remodeling of the gut microbiome.	2017	He *et al.* [64]
Human	10-16 years old	Six patients with drug-resistant epilepsy.	Antibiotic treatments	Patients attained temporary seizure freedom during antibiotic treatment.	2018	Braakman and van Ingen [65]
Human	Mean age 44 years old	45 patients with drug-resistant epilepsy.	Probiotic treatments	28.9% of all patients displayed a greater than 50% reduction in the number of seizures. A significant improvement was observed in patients' quality of life. Probiotics may be an option for supplementary therapy.	2018	Gomez-Eguilaz *et al.* [66]

were noted in individuals with fewer seizures (a maximum of four seizures in 1 year). Meanwhile, the drug-sensitive patients displayed gut microbiome populations comparable to those of the control group. These results indicate the possible involvement of dysbiosis in the development of drug-resistant epilepsy; hence, a novel approach to the treatment of drug-resistant epilepsy might involve restoration of a healthy gut microbial population [62].

Another recent study examined whether rats can be made susceptible to epilepsy by induced dysbiosis resulting from chronic restraint stress [63]. Progression of the illness was accelerated, and the duration of the resulting seizures increased, not only in chronically stressed individuals but also in so-called sham-stressed rats (previously healthy rats given a transplanted microbiome population from stressed individuals) [63]. In addition, the proepileptic impacts of restraint stress were reversed when the chronically stressed individuals were given a transplanted microbiome from the sham-stressed rats [63]. These observations provide direct evidence for a link between alterations in the gut microbiome (specifically caused by chronic stress) and an enhanced predisposition to epilepsy [63].

Although the clinical application of microbiota in the treatment of brain diseases has not been widely investigated, one strategy with positive potential is restoration of the gut microbiota via faecal microbiota transplantation (FMT) [67]. This approach was recently investigated in a case study involving the treatment of long-term (17-year) epilepsy in a patient with Crohn's disease (CD) [64]. During the 20-month follow-up period, the FMT treatment was effective in both alleviating intestinal symptoms and preventing the recurrence of seizures after the withdrawal of antiepileptic drugs [68]. These observations emphasise the contribution of the microbiota-gut-brain axis and support the novel concept of remodeling the gut microbiota in the treatment for epilepsy.

In another contemporary study, the effects of antibiotics upon the frequency of seizures were investigated in six interesting drug-resistant epilepsy cases [65]. Short-term (2-week) cessation of seizures was observed after the antibiotic treatments, again demonstrating the probable role of the gut microbiota in the development and symptoms of epilepsy and supporting the idea that drug-resistant cases could be treated by adjusting the gut microbiota to disrupt the unfavourable microbiota [65]. It implies that such an intervention could directly affect the frequency of seizures by influencing the gut-brain interactions.

A group of 45 drug-resistant epileptic patients were given a 4-month course of probiotics in a prospective study in

which the levels of interleukin 6, γ-aminobutyric acid, and CD-14 were evaluated along with quality of life (QOLIE-10) and the number of seizures before and after the treatment [66]. Intention-to-treat analysis demonstrated the 50% reduction in seizures required by clinical trials in 28.9% of patients, along with a notable improvement in quality of life [66]. These results demonstrate the potential use of probiotics as a safe supplementary treatment to enhance control of seizures, and hence the quality of life, in patients with drug-resistant epilepsy.

3. Links between the Ketogenic Diet, Microbiota, and Epilepsy

3.1. Dietary Adjustment of Gut Microbiota. Numerous studies have shown that the composition of the diet is a key factor in determining the composition of the gut microbiome in various conditions of health and at various stages of life [69]. Indeed, changes in diet seem to have the greatest influence upon the gut microbiota, being responsible for much of the general variation in the gut microbiota structure as well as modifying disease susceptibility by either triggering or circumventing dysbiosis [70–72]. The adjustment of gut microbiota via dietary composition primarily involves altering the proportion of dietary fibre, also referred to as microbiota-accessible carbohydrates (MACs), along with dietary protein and fat [73]. The gut microbiota is dependent upon dietary fibre for energy and sustenance. Moreover, the composition of the diet, especially fibre, seems to be a key factor in gut bacterial function, ecology, and diversity [74]. In addition, dietary protein provides the primary source of nitrogen required for the growth of colonic microbes and is critical for carbohydrate assimilation and generation of valuable molecules [75], such as short-chain fatty acids (SCFAs) [76]. However, diets that are high in protein have also been found to raise the levels of harmful metabolites in faeces and are linked to conditions such as cancer and inflammatory bowel disease [77]. Finally, the fat content provides the diet with a high caloric value; 40% to 55% of calories in the Western diet are provided by lipids. It has been shown that the diurnal structural and functional characteristics of the gut microbiota are influenced by a high-fat diet [78]. In addition, it was recently shown that alteration of the gut microbiota by ω-3 fatty acids can reduce chronic inflammation and prevent weight gain [79].

3.2. Recent Advances in the Treatment of Epilepsy by Ketogenic Diet. The ketogenic diet (KD) restricts the caloric intake to 10% to 25% and consists of more than 90% fat, almost no sugar, and just sufficient amounts of vitamins, minerals, and proteins [80]. Despite the known harmful effects of excess dietary fat, the KD has been used as a therapeutic diet since the early 20th century, and its application in the treatment of epilepsy was inspired by age-old observations regarding the positive effects of fasting [20]. The therapeutic use of the KD has regained popularity in recent years to become the focus of considerable scientific investigation [81]. To increase adherence to the diet, the modern KD has been adapted according to scientifically established variations that make it more appetising and less restrictive (e.g., the modified Atkins diet and the low glycaemic index diet) [82]. The antiepileptic effects of these KD-type diets are presently used in the treatment of drug-resistant adult and child patients [6, 83–87]. In addition to their benefit for certain forms of epilepsy, the KD appears to have some positive effects on other neurological conditions, such as migraine, glaucoma, multiple sclerosis, Parkinson's disease, and Alzheimer's disease [81, 88–90].

Although the positive effects of the KD in reducing epileptic seizures are well proven, the precise mechanism by which this occurs has yet to be fully elucidated. Nevertheless, the recent literature indicates some advances in this topic [87, 91, 92], including the implication of the gut microbiota alongside changes in the functioning of the mitochondria, alteration of neurotransmitter release and neuron function by ketone bodies, and antiepileptic effects of glucose stabilisation and/or fatty acids [91, 93]. Nevertheless, more research is needed to increase our understanding of these potential mechanisms [94].

3.3. Involvement of Gut Microbiota in the Treatment of Epilepsy by KD. Few studies have dealt with the involvement of the gut microbiota in the treatment of epilepsy with the KD [93, 95]. Examination of the composition and characteristics of the gut microbiota during KD treatment of epilepsy has indicated the potential mechanism presented in Figure 1.

Remarkable findings relating to the microbiota- and ketogenic diet-dependent protection from seizures were reported by Olson *et al.* after a study in which two mouse models of refractory epilepsy were used to demonstrate mediation of the protective effect by the gut microbiota [95]. The susceptibility to and incidence of seizures were found to increase in individuals on the KD diet when given a high-dose antibiotic treatment that led to depletion of the microbiota. However, the negative effect of the antibiotic treatment was then reversed as the gut was recolonised with bacteria. The diversity of gut microbiota was decreased, whilst the relative abundance of *Parabacteroides* and *Akkermansia muciniphila* increased, during the KD diet; hence, these specific changes may play a part in the protection from seizure activity observed. Furthermore, these changes were linked to increased bulk and glutamate levels of gamma-aminobutyric acid (GABA) in the hippocampus.

Following on from this, Hampton suggested that the antiseizure effects of the KD could be attributed to gut microbes. This study was the first to demonstrate the direct involvement of the microbiota in providing the antiseizure effects of the KD in mice [96]. This study also revealed the potential cellular and molecular mechanisms by which interactions between specific bacteria modulate the peripheral metabolites that influence the levels of hippocampal neurotransmitters [96]. Further research is warranted to establish whether the KD leads to comparable effects upon brain metabolites and amino acids in humans and to answer other intriguing questions, such as whether the role of the hippocampus in childhood epilepsy is comparable in mice and in

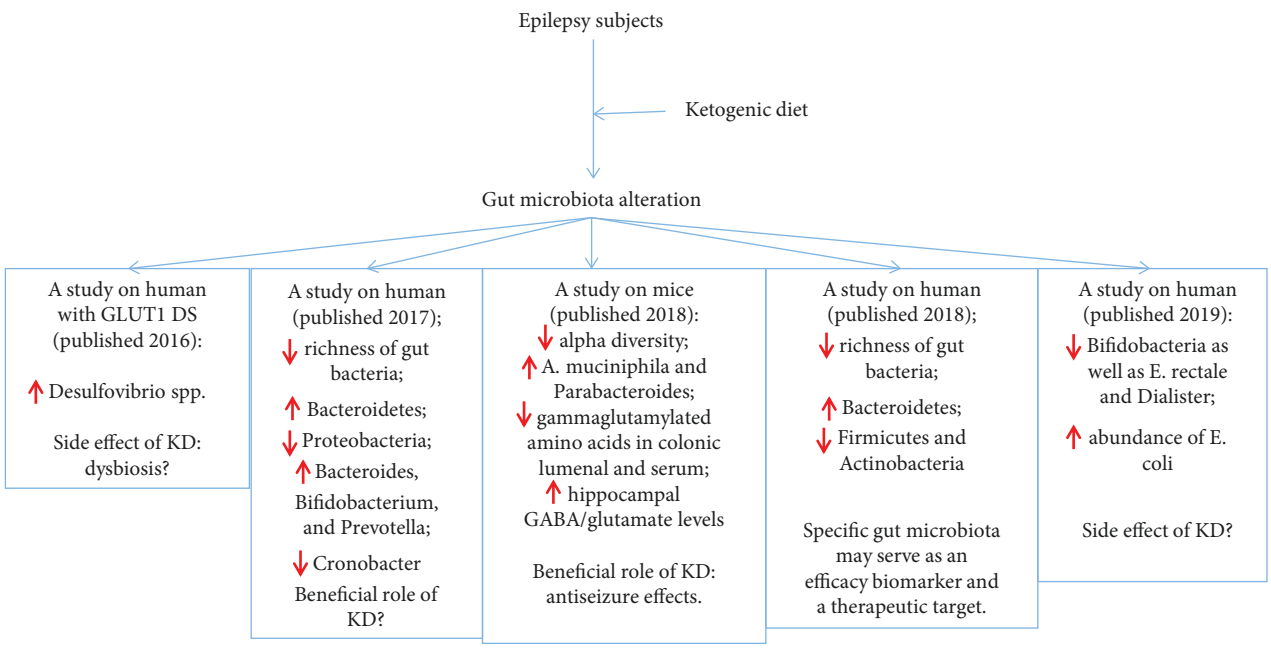

Figure 1: Possible role of gut microbiota in recent studies on the effects of the ketogenic diet on epilepsy patients. GLUT1 DS: Glucose Transporter 1 Deficiency Syndrome; KD: ketogenic diet.

humans [21]. The study's findings could also be relevant to other conditions that respond to the KD, such as Alzheimer's disease, cancer, autism spectrum disorder, and metabolic syndrome [93, 97, 98]. Meanwhile, a study of the effects of the KD upon children with drug-resistant epilepsy indicated a decrease in the diversity of gut microbiota after 1 week, with a decrease in the levels of the phylum Proteobacteria and an increase in the levels of the phylum Bacteroidetes [61]. At the genus level, a decrease in Cronobacter was noted alongside increases in *Prevotella*, *Bifidobacterium*, and *Bacteroides*. The microbiota in epileptic infants was found to differ from that of healthy controls and was also shown to change significantly, with increases in beneficial bacteria and decreases in pathogenic bacteria, in response to the KD [61]. Hence, the study suggested that the KD could quickly modify the gut microbiota and reduce the frequency of seizures in infants with drug-resistant epilepsy.

In a separate investigation, the faecal microbiota profiles of children with resistant epilepsy revealed decreased diversity, with decreased levels of Firmicutes and increased levels of Bacteroidetes after 6 months of KD treatment [99]. The subjects of this study showed various seizure-reduction responses, and those who failed to respond had enhanced levels of *Alistipes*, *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae*, and *Rikenellaceae* relative to those who responded to treatment [99]. This suggests that the KD may have varied efficacy with respect to altering the composition of the gut microbiota and that specific microbiota may provide both possible therapeutic targets and biomarkers for the efficacy of the treatment in individuals with resistant epilepsy [99].

The above-mentioned study was critiqued by E. Spinelli and R. Blackford, who raised the following questions:

(i) How does the gut bacteria influence the onset of epilepsy

(ii) Is it possible to use the composition of the gut microbiota as a marker to monitor the effectiveness of treatment, as has been done in mouse models

(iii) Can the bacterial composition be modified as a therapeutic approach

These commenters also point to the need for a large-scale multicentre investigation to build upon the above observations and to better demonstrate whether a possible microbe-based treatment is a rational choice in paediatric refractory epilepsy [100].

In another recent study, six individuals with GLUT1 deficiency syndrome were asked to collect faecal samples before and after 3 months of KD treatment to compare the microbiota compositions [101]. *Bacteroidetes*, *Bifidobacterium spp.*, *Clostridium cluster* XIV, *Clostridium perfringens*, *Desulfovibrio spp.*, *Enterobacteriaceae*, *Faecalibacterium prausnitzii*, *Firmicutes*, and *Lactobacillus spp.* were quantified by reverse-transcription polymerase chain reaction (RT-PCR). The faecal microbial profiles demonstrated statistically significant enhancement in the levels of *Desulfovibrio spp.*, a group of bacteria believed to contribute to aggravated inflammation of the gut mucosa resulting from ingestion of animal fats [101].

In view of the above research, a future prospective investigation on alterations in the gut microbiota of epileptic children subjected to KD treatment is warranted. In addition, it may be logical to propose an empirical test of the potential for prebiotics or probiotics to reestablish the optimum

ecological balance of the intestinal microbiota in individuals whose faecal samples indicate dysbiosis [102].

A study in 12 children with drug-resistant epilepsy was recently published online [103]. Faecal samples were collected before and after 3 months of KD treatment, whilst the parents functioned as a diet control group. Alterations in both the taxonomic and functional profiles were detected via shotgun metagenomic DNA sequencing. The treatment resulted in notable reductions in the relative abundances of *Bifidobacteria*, *Dialister*, and *Eubacterium rectale* along with an increase in the relative abundance of *Escherichia coli* [103]. Alterations in 29 SEED subsystems were indicated by functional analysis, including the decline of seven carbohydrate metabolism pathways. The analysis demonstrated that the *Bifidobacteria* and *E. coli* contributed significantly to the functional changes [103]. The study expressed misgivings surrounding the effects of the KD upon the gut microbiota and the patients' general health because the relative abundance of beneficial fibre-consuming bacteria decreased in response to the KD treatment. Consequently, additional research is needed to establish whether these specific alterations are essential for the therapeutic impact of KD treatment.

The KD incorporates a range of mechanisms that lead to decreased neuronal excitability, including alteration of the gut microbiota [104]. Further classification of the specific mechanisms could contribute to the replacement of the strict KD treatment by dietary supplements, such as probiotics and/or prebiotics. Moreover, recognition of the disease-altering characteristics of KD therapy could provide hope for a long-term therapeutic effect that could continue even after the diet has ended.

4. Outlook

Recent studies have demonstrated a close link between epilepsy and the gut microbiome. Moreover, the mechanism behind the antiseizure effects of the KD in epileptic patients may be contributed by the gut microbiota. There is therefore much potential in optimising the KD to promote specific microbes as a neuroprotective treatment for drug-resistant epilepsy. Nevertheless, numerous questions must still be fully addressed, including the specific mechanism by which the gut microbiota influences the onset of epilepsy and the possible clinical applications of findings relating to the function of gut microbiota in the KD treatment of epilepsy. Moreover, these questions can only be answered with the establishment of large multicentre research efforts. The KD has potential benefits not only in epilepsy but also in numerous other disorders linked to changes in GABA, including Alzheimer's disease, anxiety, autism, Parkinson's disease, and schizophrenia. Hence, a broad spectrum of grave health issues could be positively affected by the development of a neuroprotective therapy involving microbes.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Yuying Fan finished the first draft of the manuscript; Hua Wang, Xueyan Liu, Junmei Zhang, and Gang Liu critically revised the manuscript; all the authors read, revised, and approved the submission of the manuscript.

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References

[1] S. D. Shukla, K. F. Budden, R. Neal, and P. M. Hansbro, "Microbiome effects on immunity, health and disease in the lung," *Clinical & Translational Immunology*, vol. 6, no. 3, p. e133, 2017.

[2] K. Baruch and M. Schwartz, "Circulating monocytes in between the gut and the mind," *Cell Stem Cell*, vol. 18, no. 6, pp. 689–691, 2016.

[3] C. Mu, Y. Yang, and W. Zhu, "Gut microbiota: the brain peacekeeper," *Frontiers in Microbiology*, vol. 7, 2016.

[4] T. G. Dinan and J. F. Cryan, "Gut-brain axis in 2016: brain-gut-microbiota axis - mood, metabolism and behaviour," *Nature Reviews Gastroenterology & Hepatology*, vol. 14, no. 2, pp. 69-70, 2017.

[5] P. J. Kennedy, J. F. Cryan, T. G. Dinan, and G. Clarke, "Kynurenine pathway metabolism and the microbiota-gut-brain axis," *Neuropharmacology*, vol. 112, pp. 399–412, 2017, Pt B.

[6] L. P. de Brito Sampaio, "Ketogenic diet for epilepsy treatment," *Arquivos de Neuro-Psiquiatria*, vol. 74, no. 10, pp. 842–848, 2016.

[7] F. G. Bournissen, M. E. Moretti, D. N. Juurlink, G. Koren, M. Walker, and Y. Finkelstein, "Polymorphism of the MDR1/ABCB1 C3435T drug-transporter and resistance to anticonvulsant drugs: a meta-analysis," *Epilepsia*, vol. 50, no. 4, pp. 898–903, 2009.

[8] H. Meng, G. Guo, J. Ren, H. Zhou, Y. Ge, and Y. Guo, "Effects of ABCB1 polymorphisms on plasma carbamazepine concentrations and pharmacoresistance in Chinese patients with epilepsy," *Epilepsy & Behavior*, vol. 21, no. 1, pp. 27–30, 2011.

[9] K. Wang, H. Li, H. Wang, J.-h. Wang, F. Song, and Y. Sun, "Irisin exerts neuroprotective effects on cultured neurons by regulating astrocytes," *Mediators of Inflammation*, vol. 2018, 7 pages, 2018.

[10] M. S. Ong, I. S. Kohane, T. Cai, M. P. Gorman, and K. D. Mandl, "Population-level evidence for an autoimmune etiology of epilepsy," *JAMA Neurology*, vol. 71, no. 5, pp. 569–574, 2014.

[11] A. K. Yeshokumar and C. A. Pardo, "Autoimmune epilepsies," *Seminars in Pediatric Neurology*, vol. 24, no. 3, pp. 161–167, 2017.

[12] Y. Li, B. Jing, H. Liu et al., "Frequency-dependent changes in the amplitude of low-frequency fluctuations in mild cognitive impairment with mild depression," *Journal of Alzheimer's Disease*, vol. 58, no. 4, pp. 1175–1187, 2017.

[13] H. Wekerle, "Brain autoimmunity and intestinal microbiota: 100 trillion game changers," *Trends in Immunology*, vol. 38, no. 7, pp. 483–497, 2017.

[14] Y. Fan and J. Zhang, "Dietary modulation of intestinal microbiota: future opportunities in experimental autoimmune encephalomyelitis and multiple sclerosis," *Frontiers in Microbiology*, vol. 10, p. 740, 2019.

[15] C. H. Chen, C. L. Lin, and C. H. Kao, "Irritable Bowel Syndrome Increases the Risk of Epilepsy," *Medicine*, vol. 94, no. 36, p. e1497, 2015.

[16] M. A. Jackson, S. Verdi, M. E. Maxan et al., "Gut microbiota associations with common diseases and prescription medications in a population-based cohort," *Nature Communications*, vol. 9, no. 1, p. 2655, 2018.

[17] L. A. David, C. F. Maurice, R. N. Carmody et al., "Diet rapidly and reproducibly alters the human gut microbiome," *Nature*, vol. 505, no. 7484, pp. 559–563, 2014.

[18] A. Clark and N. Mach, "Exercise-induced stress behavior, gut-microbiota-brain axis and diet: a systematic review for athletes," *Journal of the International Society of Sports Nutrition*, vol. 13, no. 1, 2016.

[19] E. H. Kossoff, B. A. Zupec-Kania, S. Auvin et al., "Optimal clinical management of children receiving dietary therapies for epilepsy: updated recommendations of the International Ketogenic Diet Study Group," *Epilepsia Open*, vol. 3, no. 2, pp. 175–192, 2018.

[20] K. Martin, C. F. Jackson, R. G. Levy, and P. N. Cooper, "Ketogenic diet and other dietary treatments for epilepsy," *Cochrane Database of Systematic Reviews*, vol. 2, article CD001903, 2016.

[21] A. Lutas and G. Yellen, "The ketogenic diet: metabolic influences on brain excitability and epilepsy," *Trends in Neurosciences*, vol. 36, no. 1, pp. 32–40, 2013.

[22] S. Reddel, L. Putignani, and F. Del Chierico, "The impact of low-FODMAPs, gluten-free, and ketogenic diets on gut microbiota modulation in pathological conditions," *Nutrients*, vol. 11, no. 2, p. 373, 2019.

[23] I. Sekirov and B. B. Finlay, "The role of the intestinal microbiota in enteric infection," *The Journal of Physiology*, vol. 587, no. 17, pp. 4159–4167, 2009.

[24] A. Cabinian, D. Sinsimer, M. Tang et al., "Gut symbiotic microbes imprint intestinal immune cells with the innate receptor SLAMF4 which contributes to gut immune protection against enteric pathogens," *Gut*, vol. 67, no. 5, pp. 847–859, 2018.

[25] M. Candela, E. Biagi, S. Turroni, S. Maccaferri, P. Figini, and P. Brigidi, "Dynamic efficiency of the human intestinal microbiota," *Critical Reviews in Microbiology*, vol. 41, no. 2, pp. 165–171, 2015.

[26] M. Shapira, "Gut microbiotas and host evolution: scaling up symbiosis," *Trends in Ecology & Evolution*, vol. 31, no. 7, pp. 539–549, 2016.

[27] E. A. Mayer, R. Knight, S. K. Mazmanian, J. F. Cryan, and K. Tillisch, "Gut microbes and the brain: paradigm shift in neuroscience," *Journal of Neuroscience*, vol. 34, no. 46, pp. 15490–15496, 2014.

[28] C. Schmidt, "Mental health: thinking from the gut," *Nature*, vol. 518, no. 7540, pp. S12–S14, 2015.

[29] P. A. Smith, "The tantalizing links between gut microbes and the brain," *Nature*, vol. 526, no. 7573, pp. 312–314, 2015.

[30] T. Jenkins, J. Nguyen, K. Polglaze, and P. Bertrand, "Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis," *Nutrients*, vol. 8, no. 1, p. 56, 2016.

[31] J. F. Cryan and T. G. Dinan, "Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour," *Nature Reviews Neuroscience*, vol. 13, no. 10, pp. 701–712, 2012.

[32] R. D. Heijtz, S. Wang, F. Anuar et al., "Normal gut microbiota modulates brain development and behavior," *Proceedings of the National Academy of Sciences*, vol. 108, no. 7, pp. 3047–3052, 2011.

[33] S. M. O'Mahony, G. Clarke, Y. E. Borre, T. G. Dinan, and J. F. Cryan, "Serotonin, tryptophan metabolism and the brain-gut-microbiome axis," *Behavioural Brain Research*, vol. 277, pp. 32–48, 2015.

[34] P. Luczynski, K.-A. McVey Neufeld, C. S. Oriach, G. Clarke, T. G. Dinan, and J. F. Cryan, "Growing up in a bubble: using germ-free animals to assess the influence of the gut microbiota on brain and behavior," *International Journal of Neuropsychopharmacology*, vol. 19, no. 8, p. pyw020, 2016.

[35] K. C. Bauer, K. E. Huus, and B. B. Finlay, "Microbes and the mind: emerging hallmarks of the gut microbiota-brain axis," *Cellular Microbiology*, vol. 18, no. 5, pp. 632–644, 2016.

[36] Y. Al Omran and Q. Aziz, "The brain-gut axis in health and disease," *Advances in Experimental Medicine and Biology*, vol. 817, pp. 135–153, 2014.

[37] S. Masino, M. Kawamura Jr., C. Wasser, L. Pomeroy, and D. Ruskin, "Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity," *Current Neuropharmacology*, vol. 7, no. 3, pp. 257–268, 2009.

[38] S. Fazel, A. Wolf, N. Langstrom, C. R. Newton, and P. Lichtenstein, "Premature mortality in epilepsy and the role of psychiatric comorbidity: a total population study," *Lancet*, vol. 382, no. 9905, pp. 1646–1654, 2013.

[39] O. Devinsky, E. Marsh, D. Friedman et al., "Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial," *The Lancet Neurology*, vol. 15, no. 3, pp. 270–278, 2016.

[40] O. Bennett-Back, A. Keren, and N. Zelnik, "Attention-deficit hyperactivity disorder in children with benign epilepsy and their siblings," *Pediatric Neurology*, vol. 44, no. 3, pp. 187–192, 2011.

[41] T. A. Pollak, T. R. Nicholson, J. D. C. Mellers, A. Vincent, and A. S. David, "Epilepsy-related psychosis: a role for autoimmunity?," *Epilepsy & Behavior*, vol. 36, pp. 33–38, 2014.

[42] A. M. Quek, J. W. Britton, A. McKeon et al., "Autoimmune epilepsy: clinical characteristics and response to immunotherapy," *Archives of Neurology*, vol. 69, no. 5, pp. 582–593, 2012.

[43] G. Gozubatik-Celik, C. Ozkara, C. Ulusoy et al., "Anti-neuronal autoantibodies in both drug responsive and resistant focal seizures with unknown cause," *Epilepsy Research*, vol. 135, pp. 131–136, 2017.

[44] P. Borusiak, U. Bettendorf, G. Wiegand et al., "Autoantibodies to neuronal antigens in children with focal epilepsy and no prima facie signs of encephalitis," *European Journal of Paediatric Neurology*, vol. 20, no. 4, pp. 573–579, 2016.

[45] A. Vincent and P. B. Crino, "Systemic and neurologic autoimmune disorders associated with seizures or epilepsy," *Epilepsia*, vol. 52, pp. 12–17, 2011, Suppl 3.

[46] G. Avanzini, A. Depaulis, A. Tassinari, and M. de Curtis, "Do seizures and epileptic activity worsen epilepsy and deteriorate cognitive function?," *Epilepsia*, vol. 54, pp. 14–21, 2013.

[47] S. Weckhuysen, S. Mandelstam, A. Suls et al., "KCNQ2 encephalopathy: emerging phenotype of a neonatal epileptic encephalopathy," *Annals of Neurology*, vol. 71, no. 1, pp. 15–25, 2012.

[48] H. C. Mefford, H. Muhle, P. Ostertag et al., "Genome-wide copy number variation in epilepsy: novel susceptibility loci in idiopathic generalized and focal epilepsies," *PLoS Genetics*, vol. 6, no. 5, article e1000962, 2010.

[49] H. Tlaskalová-Hogenová, R. Štěpánková, H. Kozáková et al., "The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases," *Cellular & Molecular Immunology*, vol. 8, no. 2, pp. 110–120, 2011.

[50] J. L. Round and S. K. Mazmanian, "The gut microbiota shapes intestinal immune responses during health and disease," *Nature Reviews Immunology*, vol. 9, no. 5, pp. 313–323, 2009.

[51] Y. Belkaid and T. W. Hand, "Role of the microbiota in immunity and inflammation," *Cell*, vol. 157, no. 1, pp. 121–141, 2014.

[52] C. L. Boulange, A. L. Neves, J. Chilloux, J. K. Nicholson, and M. E. Dumas, "Impact of the gut microbiota on inflammation, obesity, and metabolic disease," *Genome Medicine*, vol. 8, no. 1, p. 42, 2016.

[53] B. B. Yoo and S. K. Mazmanian, "The enteric network: interactions between the immune and nervous systems of the gut," *Immunity*, vol. 46, no. 6, pp. 910–926, 2017.

[54] T. C. Fung, C. A. Olson, and E. Y. Hsiao, "Interactions between the microbiota, immune and nervous systems in health and disease," *Nature Neuroscience*, vol. 20, no. 2, pp. 145–155, 2017.

[55] K. Wang, F. Song, H. Wang, J.-h. Wang, and Y. Sun, "Quetiapine attenuates the neuroinflammation and executive function deficit in streptozotocin-induced diabetic mice," *Mediators Inflamm*, vol. 2019, pp. 1–8, 2019.

[56] C. De Filippo, D. Cavalieri, M. Di Paola et al., "Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa," *Proceedings of the National Academy of Sciences*, vol. 107, no. 33, pp. 14691–14696, 2010.

[57] C. Wathen and D. Janigro, "IL-1β associations with posttraumatic epilepsy development: A genetics and biomarker cohort study," *Epilepsia*, vol. 55, no. 8, p. 1313, 2014.

[58] F. Ye, X. Q. Chen, G. S. Bao, Y. Hua, Z. D. Wang, and Y. C. Bao, "Effect of topiramate on interleukin 6 expression in the hippocampus of amygdala-kindled epileptic rats," *Experimental and Therapeutic Medicine*, vol. 7, no. 1, pp. 223–227, 2014.

[59] K. Atarashi, T. Tanoue, M. Ando et al., "Th17 cell induction by adhesion of microbes to intestinal epithelial cells," *Cell*, vol. 163, no. 2, pp. 367–380, 2015.

[60] L. Y. Mao, J. Ding, W. F. Peng et al., "Interictal interleukin-17A levels are elevated and correlate with seizure severity of epilepsy patients," *Epilepsia*, vol. 54, no. 9, pp. e142–e145, 2013.

[61] G. Xie, Q. Zhou, C. Z. Qiu et al., "Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy," *World Journal of Gastroenterology*, vol. 23, no. 33, pp. 6164–6171, 2017.

[62] A. Peng, X. Qiu, W. Lai et al., "Altered composition of the gut microbiome in patients with drug-resistant epilepsy," *Epilepsy Research*, vol. 147, pp. 102–107, 2018.

[63] J. S. Medel-Matus, D. Shin, E. Dorfman, R. Sankar, and A. Mazarati, "Facilitation of kindling epileptogenesis by chronic stress may be mediated by intestinal microbiome," *Epilepsia Open*, vol. 3, no. 2, pp. 290–294, 2018.

[64] Z. He, B. T. Cui, T. Zhang et al., "Fecal microbiota transplantation cured epilepsy in a case with Crohn's disease: the first report," *World Journal of Gastroenterology*, vol. 23, no. 19, pp. 3565–3568, 2017.

[65] H. M. H. Braakman and J. van Ingen, "Can epilepsy be treated by antibiotics?," *Journal of Neurology*, vol. 265, no. 8, pp. 1934–1936, 2018.

[66] M. Gomez-Eguilaz, J. L. Ramon-Trapero, L. Perez-Martinez, and J. R. Blanco, "The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: a pilot study," *Beneficial Microbes*, vol. 9, no. 6, pp. 875–881, s.

[67] M. Kuitunen, K. Kukkonen, K. Juntunen-Backman et al., "Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort," *Journal of Allergy and Clinical Immunology*, vol. 123, no. 2, pp. 335–341, 2009.

[68] P. Lahtinen, E. Mattila, V. J. Anttila et al., "Faecal microbiota transplantation in patients with Clostridium difficile and significant comorbidities as well as in patients with new indications: a case series," *World Journal of Gastroenterology*, vol. 23, no. 39, pp. 7174–7184, 2017.

[69] M. J. Claesson, I. B. Jeffery, S. Conde et al., "Gut microbiota composition correlates with diet and health in the elderly," *Nature*, vol. 488, no. 7410, pp. 178–184, 2012.

[70] L. Gentschew and L. R. Ferguson, "Role of nutrition and microbiota in susceptibility to inflammatory bowel diseases," *Molecular Nutrition & Food Research*, vol. 56, no. 4, pp. 524–535, 2012.

[71] M. S. Desai, A. M. Seekatz, N. M. Koropatkin et al., "A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility," *Cell*, vol. 167, no. 5, pp. 1339–1353.e21, 2016.

[72] K. Brown, D. DeCoffe, E. Molcan, and D. L. Gibson, "Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease," *Nutrients*, vol. 4, no. 8, pp. 1095–1119, 2012.

[73] E. D. Sonnenburg and J. L. Sonnenburg, "Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates," *Cell Metabolism*, vol. 20, no. 5, pp. 779–786, 2014.

[74] J. Tan, C. McKenzie, P. J. Vuillermin et al., "Dietary fiber and bacterial SCFA enhance oral tolerance and protect against food allergy through diverse cellular pathways," *Cell Reports*, vol. 15, no. 12, pp. 2809–2824, 2016.

[75] C. K. Yao, J. G. Muir, and P. R. Gibson, "Review article: insights into colonic protein fermentation, its modulation and potential health implications," *Alimentary Pharmacology & Therapeutics*, vol. 43, no. 2, pp. 181–196, 2016.

[76] C. S. Reigstad, C. E. Salmonson, J. F. Rainey III et al., "Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells," *The FASEB Journal*, vol. 29, no. 4, pp. 1395–1403, 2015.

[77] N. Zhang, Z. Ju, and T. Zuo, "Time for food: the impact of diet on gut microbiota and human health," *Nutrition*, vol. 51-52, pp. 80–85, 2018.

[78] V. Leone, S. M. Gibbons, K. Martinez et al., "Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism," *Cell Host Microbe*, vol. 17, no. 5, pp. 681–689, 2015.

[79] K. Kaliannan, B. Wang, X. Y. Li, A. K. Bhan, and J. X. Kang, "Omega-3 fatty acids prevent early-life antibiotic exposure-induced gut microbiota dysbiosis and later-life obesity," *International Journal of Obesity*, vol. 40, no. 6, pp. 1039–1042, 2016.

[80] C. Kosinski and F. Jornayvaz, "Effects of ketogenic diets on cardiovascular risk factors: evidence from animal and human studies," *Nutrients*, vol. 9, no. 5, p. 517, 2017.

[81] T. Walczyk and J. Y. Wick, "The ketogenic diet: making a comeback," *The Consultant Pharmacist*, vol. 32, no. 7, pp. 388–396, 2017.

[82] E. H. Kossoff, "The ketogenic diet: an appropriate first-line therapy?," *Expert Review of Neurotherapeutics*, vol. 10, no. 6, pp. 843–845, 2010.

[83] S. Auvin, "Non-pharmacological medical treatment in pediatric epilepsies," *Revue Neurologique*, vol. 172, no. 3, pp. 182–185, 2016.

[84] S. Park, E. J. Lee, S. Eom, H. C. Kang, J. S. Lee, and H. D. Kim, "Ketogenic diet for the management of epilepsy associated with tuberous sclerosis complex in children," *Journal of Epilepsy Research*, vol. 7, no. 1, pp. 45–49, 2017.

[85] T. J. Williams and M. C. Cervenka, "The role for ketogenic diets in epilepsy and status epilepticus in adults," *Clinical Neurophysiology Practice*, vol. 2, pp. 154–160, 2017.

[86] S. J. Koppel and R. H. Swerdlow, "Neuroketotherapeutics: a modern review of a century-old therapy," *Neurochemistry International*, vol. 117, pp. 114–125, 2018.

[87] Y. Zhang, J. Xu, K. Zhang, W. Yang, and B. Li, "The anticonvulsant effects of ketogenic diet on epileptic seizures and potential mechanisms," *Current Neuropharmacology*, vol. 16, no. 1, pp. 66–70, 2017.

[88] I. Y. Choi, L. Piccio, P. Childress et al., "A diet mimicking fasting promotes regeneration and reduces autoimmunity and multiple sclerosis symptoms," *Cell Reports*, vol. 15, no. 10, pp. 2136–2146, 2016.

[89] P. Barbanti, L. Fofi, C. Aurilia, G. Egeo, and M. Caprio, "Ketogenic diet in migraine: rationale, findings and perspectives," *Neurological Sciences*, vol. 38, Suppl 1, pp. 111–115, 2017.

[90] A. Verrotti, G. Iapadre, S. Pisano, and G. Coppola, "Ketogenic diet and childhood neurological disorders other than epilepsy: an overview," *Expert Review of Neurotherapeutics*, vol. 17, no. 5, pp. 461–473, 2017.

[91] D. Boison, "New insights into the mechanisms of the ketogenic diet," *Current Opinion in Neurology*, vol. 30, no. 2, pp. 187–192, 2017.

[92] N. A. Youngson, M. J. Morris, and J. W. O. Ballard, "The mechanisms mediating the antiepileptic effects of the ketogenic diet, and potential opportunities for improvement with metabolism-altering drugs," *Seizure*, vol. 52, pp. 15–19, 2017.

[93] C. Newell, M. R. Bomhof, R. A. Reimer, D. S. Hittel, J. M. Rho, and J. Shearer, "Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder," *Molecular Autism*, vol. 7, no. 1, p. 37, 2016.

[94] B. G. Allen, S. K. Bhatia, C. M. Anderson et al., "Ketogenic diets as an adjuvant cancer therapy: history and potential mechanism," *Redox Biology*, vol. 2, pp. 963–970, 2014.

[95] C. A. Olson, H. E. Vuong, J. M. Yano, Q. Y. Liang, D. J. Nusbaum, and E. Y. Hsiao, "The gut microbiota mediates the anti-seizure effects of the ketogenic diet," *Cell*, vol. 173, no. 7, pp. 1728–1741.e13, 2018.

[96] T. Hampton, "Gut microbes may account for the anti-seizure effects of the ketogenic diet," *JAMA*, vol. 320, no. 13, p. 1307, 2018.

[97] S. T. Henderson, J. L. Vogel, L. J. Barr, F. Garvin, J. J. Jones, and L. C. Costantini, "Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer's disease: a randomized, double-blind, placebo-controlled, multicenter trial," *Nutrition & Metabolism*, vol. 6, no. 1, p. 31, 2009.

[98] B. G. Allen, S. K. Bhatia, J. M. Buatti et al., "Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts," *Clinical Cancer Research*, vol. 19, no. 14, pp. 3905–3913, 2013.

[99] Y. Zhang, S. Zhou, Y. Zhou, L. Yu, L. Zhang, and Y. Wang, "Altered gut microbiome composition in children with refractory epilepsy after ketogenic diet," *Epilepsy Research*, vol. 145, pp. 163–168, 2018.

[100] E. Spinelli and R. Blackford, "Gut microbiota, the ketogenic diet and epilepsy," *Pediatric Neurology Briefs*, vol. 32, no. 0, p. 10, 2018.

[101] A. Ramm-Pettersen, K. O. Nakken, K. C. Haavardsholm, and K. K. Selmer, "GLUT1-deficiency syndrome: report of a four-generation Norwegian family with a mild phenotype," *Epilepsy & Behavior*, vol. 70, pp. 1–4, 2017, Pt A.

[102] A. Tagliabue, C. Ferraris, F. Uggeri et al., "Short-term impact of a classical ketogenic diet on gut microbiota in GLUT1 deficiency syndrome: a 3-month prospective observational study," *Clinical Nutrition ESPEN*, vol. 17, pp. 33–37, 2017.

[103] M. Lindefeldt, A. Eng, H. Darban et al., "The ketogenic diet influences taxonomic and functional composition of the gut microbiota in children with severe epilepsy," *npj Biofilms and Microbiomes*, vol. 5, no. 1, 2019.

[104] Q. Li and J. M. Zhou, "The microbiota-gut-brain axis and its potential therapeutic role in autism spectrum disorder," *Neuroscience*, vol. 324, pp. 131–139, 2016.

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Research Article

Galanin Protects Rat Cortical Astrocyte from Oxidative Stress: Involvement of GalR2 and pERK1/2 Signal Pathway

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The neuropeptide galanin and its receptors have been found to have protective effects on neurons. However, the role of galanin on astrocytes is still unclear. The present study is aimed at investigating the effects of galanin on the viability of cultured rat cortical astrocytes after oxidative stress induced by H_2O_2 and possible receptor and signaling mechanisms involved. Treatment of galanin had significant protective effects against H_2O_2-induced toxicity in the cultured cortical astrocytes. H_2O_2 induced an upregulation of phosphorylated extracellular signal-related kinase1/2 (pERK1/2) in astrocytes, which was suppressed by coapplication of galanin, suggesting an involvement of the pERK1/2 signal pathway in the protective effects of galanin. GalR2 has higher expression levels than GalR1 and GalR3 in the cultured cortical astrocytes, and GalR2 agonist AR-M1896 mimicked galanin effects on the astrocytes, implying that galanin protective effects mainly mediated by GalR2. Meanwhile, galanin had no effect on the A1-type transformation of rat cortical astrocytes. All those results suggest that galanin protects rat cortical astrocytes from oxidative stress by suppressing H_2O_2-induced upregulation of pERK1/2, mainly through GalR2.

1. Introduction

In the central nervous system (CNS), astrocytes contribute to maintain the homeostasis of the CNS [1]. As a component of blood-brain barrier, astrocytes serve as functional barriers that attract and restrict CNS inflammation [2]. Astrocytes keep the balance between their opposing functions of glutamate uptake and release [3, 4], providing glial cell involvement in the pathophysiology of epilepsy [5]. Astrocytes could drive seizure generation in mitochondrial epilepsy [6], even atypical astrocytes contributed to spontaneous recurrent seizures after diffuse traumatic brain injury [7]. Also, astrocytes contribute to synaptic plasticity, neuronal network oscillations, and cognitive processes, playing a role in Alzheimer's disease [8]. Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis. This process is a highly heterogeneous state; the function of astrocytes may be harmful or beneficial [9]. Thus, two different types of reactive astrocytes were termed "A1" (harmful) and "A2" (beneficial), respectively, according to neuroinflammation and ischemia [10].

Galanin, a 29-30 amino acid neuropeptide [11], is widely expressed in the CNS [12]. It is involved in many physiological and pathological functions, such as memory, epilepsy, Alzheimer's disease, and depression [13]. So far, three galanin receptors have been cloned, termed as GalR1, GalR2, and GalR3 [13]. Galanin is found coexisting with many classic neurotransmitters, such as 5-hydroxytryptamine in the dorsal raphe nucleus (DR), norepinephrine in the locus coeruleus (LC), and acetylcholine in the medial septal nuclei (MS) [12, 14]. Thus, galanin plays a cotransmission role in the CNS [15]. Many studies have showed that galanin may also have neurotrophic/neuroprotective effects in addition to its neurotransmission role. Its neuron protection functions have been proved from in vitro primary cultured hippocampal neurons to in vivo animal models and transgenic models [16–18]. The

neuronal protections of galanin are mediated mostly by GalR2 [17, 18]. However, GalR1 has recently been found to have a protective effect on neurons in the rat hippocampus and ischemic mouse brain [19, 20]. However, little research has been performed to investigate the protective role of galanin on astrocytes. Priller and colleagues found that galanin is able to induce c-fos mRNA in cultured rat astrocytes, providing evidence for the presence of functional galanin receptors on glial cells [21]. In the present study, the effects of galanin on the viability of cultured rat cortical astrocytes after H_2O_2-induced oxidative stress as well as the receptor and signaling mechanisms involved were investigated.

2. Materials and Methods

2.1. Culture of Rat Cortical Astrocytes. Astrocyte cultures were prepared from the cerebral cortex of 1-day-old neonatal Sprague Dawley rats. After decapitation, the brainstem, cerebellum, and diencephalons were removed in cold dissection buffer, the meninges were peeled off, then the brain were minced by scissors, incubated with 0.25% trypsin–EDTA at 37°C for 5 min, filtered through a 200 mesh. Cells were incubated at 37°C in a 5% CO2 for 1 hour in DMEM/F12 supplemented with 10% fetal bovine serum. The culture media were collected, and cells were resuspended in DMEM/F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and plated on poly-L-lysine-coated 75 cm^2 flasks at 37°C in a 5% CO_2 incubator. After about 2 weeks, cultures reached confluence and were shaken at 250 rpm for 18 hr at 37°C to dislodge cells adhering to the astrocyte layer, mainly oligodendrocytes. Secondary astrocyte cultures were established by trypsinizing confluent cultures and subplating onto dishes. In the present study, astrocytes were used at passage 3. When astrocytes were transformed, TNFα (30 ng/ml, MCE), IL-1α (3 ng/ml, MCE), and C1q (400 ng/ml, BioVision) were added in the medium.

2.2. Cytotoxicity Assay. Sensitivities of astrocytes to various chemicals were examined using the Cell-Counting Kit (CCK, Sigma, St. Louis, MO, USA) technique. Astrocytes were plated at a density of 5000 cells per well in 96-well plates. After 24 hr incubation at 37°C in a 5% CO_2 incubator, culture medium was replaced with new medium and drugs, incubated for an additional 24 hr. 10 μl CCK reagent was added into each well and incubated for 2 hr before reading at a wavelength of 450 nm. The drugs added into each well included several groups, vehicle, H_2O_2, H_2O_2+galanin, galanin, H_2O_2+AR-M1896, and AR-M1896. Absorbances were converted to percentages for comparison with the vehicle group.

2.3. Immunocytochemistry Staining. Cells on 25 mm poly-L lysine-coated glass coverslips were rinsed twice with PBS, pH 7.2-7.4, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, rinsed three times with PBS, incubated with PBS containing 0.3% Triton X-100 for 30 min, blocked in 10% goat serum in PBST for 1 hr, incubated with primary monoclonal anti-GFAP mouse antibody (Sigma, St. Louis, MO, USA) overnight at 4°C, rinsed three times with PBS, incubated with secondary goat anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) for 2 hr at RT, rinsed three times with PBS, mounted with glycerin, and examined under confocal microscope (Leica, USA) or inverted fluorescence microscope IX51 (Olympus, Japan).

2.4. Western Blot. Astrocytes were washed with PBS, lysed in RIPA lysate containing protease inhibitor cocktail (Applygen, China) and phosphatase inhibitor cocktail (Sigma, USA), and sonicated for 2 minutes. Cell lysates were centrifuged for 20 min at 13000 g at 4°C. Supernatant proteins were separated by SDS-PAGE on 12% gels and transferred onto PVDF membranes. After blocking with 5% nonfat milk in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 (TBS-T) for 2 hours at room temperature, blots were incubated with primary antibodies in TBS-T overnight at 4°C. The primary antibodies included rabbit anti-pERK1/2 antibody (1 : 1000, Cell Signaling Technology) and mouse anti-Gapdh antibody (1 : 10000, Sigma). Then, blots were washed with TBS-T three times and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse) (1 : 5000, China) at RT for 2 hours. Finally, the blots were rinsed and visualized using the enhanced chemiluminescence system (Thermo Fisher Scientific, Rockford, USA) according to the manufacturer's instructions. Optical densities of individual blot were quantified using the ImageJ software. Ratios of pERK1/2 to Gapdh were calculated for each sample, and fold changes were shown compared to the control group.

2.5. Reverse Transcription of mRNA. Total mRNA was isolated from culture of rat cortical astrocytes using the Qiagen RNeasy Mini Kits (Qiagen, Hilden, Germany), and mRNA was reverse transcribed using the SuperScript™ III RT reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual.

2.6. Polymerase Chain Reaction (PCR). PCR reaction was carried out using the PrimeSTAR® HS DNA Polymerase (Takara, Tokyo, Japan) under the following conditions: 2 min 98°C, 30 cycles of 10 s 98°C and 1 min 68°C, then 10 min 68°C. The primers were listed in Table 1. The identities of the PCR products were confirmed by sequencing.

2.7. Real-Time Quantitative PCR (qPCR). Real-time Quantitative PCR was carried out with SYBR Green (ABI). The total reaction system was 20 μl, 50°C 2 min, 95°C 10 min, 40 cycles for 95°C 15 sec, and 60°C 1 min. Gapdh was set as the internal parameter and the relative mRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method. The primers were listed in Table 2.

2.8. Statistical Analysis. Results were presented as means ± SEM or median (interquartile range). Data were evaluated by one-way ANOVA or nonparametric tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. H_2O_2-Induced Toxicity in Cultured Rat Cortical Astrocytes. Using CCK assay, we found that the toxic effect

Table 1: PCR primers.

Primer name	Primer sequence
Rat galanin	Forward: CACATGCCATTGACAACCAC
	Reverse: AACTCCATTATAGTGCGGACG
Rat GalR1	Forward: TCGGGACAGCAACCAAAC
	Reverse: TGCAGATGATTGAGAACCTTGG
Rat GalR2	Forward: GCCGCCATCGGGCTCATCTG
	Reverse: GTCGAGGTGCGCTCCATGCT
Rat GalR3	Forward: ACAGATCTCTTCATCCTCAACTT
	Reverse: GTGAGGTAGATGAGCAGATGTAC

Table 2: qPCR primers.

Primer name	Primer sequence
Rat iNOS	Forward: TGGAGCGAGTTGTGGATTG
	Reverse: GTGATGTCCAGGAAGTAGGTG
Rat TNFα	Forward: CTTCTGTCTACTGAACTTCGGG
	Reverse: CTACGGGCTTGTCACTCG
Rat IL-1β	Forward: GCAGGCTTCGAGATGAAC
	Reverse: GGGATTTTGTCGTTGCTTGTC
Rat Gapdh	Forward: GACCACCCAGCCCAGCAAGG
	Reverse: TCCCCAGGCCCCTCCTGTTG

of H_2O_2 on astrocyte viability was dependent on the concentration of H_2O_2 applied (Figure 1). The maximum effect of H_2O_2 was the astrocyte viability down to below 20%. Since the astrocyte viability was about 60% when 150 μM of H_2O_2 was applied, we chose this concentration to test the effects of galanin in the present study.

3.2. The Protective Effects of Galanin against H_2O_2-induced Toxicity in Cultured Rat Cortical Astrocytes. In order to investigate the protective effects of galanin against H_2O_2-induced toxicity, cultured rat cortical astrocytes were treated with vehicle, 150 μM H_2O_2, 150 μM H_2O_2+ galanin with various concentrations (1 μM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM), respectively. As shown in Figure 2(a), when the astrocytes were treated with the coadministration of galanin at 1 nM and 100 pM, the cell viabilities were higher as compared to that of the H_2O_2 treatment group significantly ($p < 0.01$). Galanin at lower (<10 pM) or higher (>10 nM) concentrations had no significant effects on cell loss induced by H_2O_2. Meanwhile, treatment with galanin alone, with various concentrations (1 μM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM) did not have any significant effects on the astrocyte viability (Figure 2(b)). After immunocytochemistry staining of GFAP, we found that the numbers of astrocyte were much less when treated with H_2O_2 compared to the control group (Figures 2(c) and 2(d)), while galanin (1 nM) rescued partly the loss of astrocytes (Figure 2(e)). Galanin alone did not change the number of astrocytes significantly (Figure 2(f)).

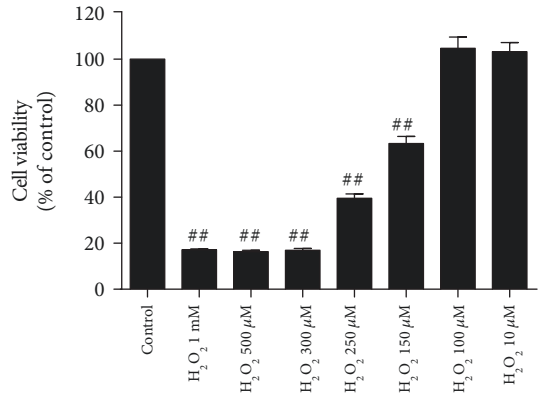

Figure 1: H_2O_2-induced toxicity in cultured rat cortical astrocytes. Cell viability was determined by CCK assay. H_2O_2 caused a dose-dependent effect on astrocyte viability. Data were presented as mean ± SE. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control group.

3.3. Involvement of pERK1/2 in the Protective Effect of Galanin against H_2O_2-Induced Toxicity. To determine the involvement of pERK1/2 in the protective effect of galanin against H_2O_2-induced toxicity, we performed the western blot experiment. The results showed that the pERK1/2 protein levels were significantly increased in the 150 μM H_2O_2 group compared to the control group ($p < 0.01$). Coapplication of 1 nM galanin significantly suppressed the H_2O_2-induced upregulation of the pERK1/2 level ($p < 0.01$). However, the pERK1/2 protein level was still higher in the H_2O_2+galanin group compared to the control group ($p < 0.05$) (Figure 3).

3.4. Involvement of GalR2 in the Protective Effect of Galanin against H_2O_2-Induced Toxicity. The mRNA expression of galanin, GalR1, GalR2, and GalR3 was detected in the cultured rat cortical astrocytes, respectively. As shown in Figure 4, expression levels of GalR2 were moderate while levels of GalR1 and GalR3 were very weak, suggesting mainly GalR2 existed in rat cortical astrocytes.

To investigate which subtype(s) of galanin receptor mediated the galanin-induced protective effects against H_2O_2 toxicity in cultured rat cortical astrocytes, GalR2 agonist AR-M1896 [22] was used. The cultured astrocytes were treated with vehicle, 150 μM H_2O_2 and H_2O_2+AR-M1896 with various concentrations (1 μM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM), respectively. As shown in Figure 5(a), coadministration with AR-M1896 at concentrations from 100 nM to 10 pM ($p < 0.01$) showed significantly higher astrocyte viability compared with H_2O_2 alone. But treatment with AR-M1896 alone at various concentrations tested above did not change astrocyte viability measured with CCK assay (Figure 5(b)). These results suggest that mainly GalR2 mediates the protective effects of galanin in cultured rat cortical astrocytes.

3.5. No Modulation Effects of Galanin on Levels of A1 transcripts in Cultured Cortical Astrocytes. It has been reported that astrocytes could be transformed to A1-type reactive astrocytes, which contribute to neuron death [10].

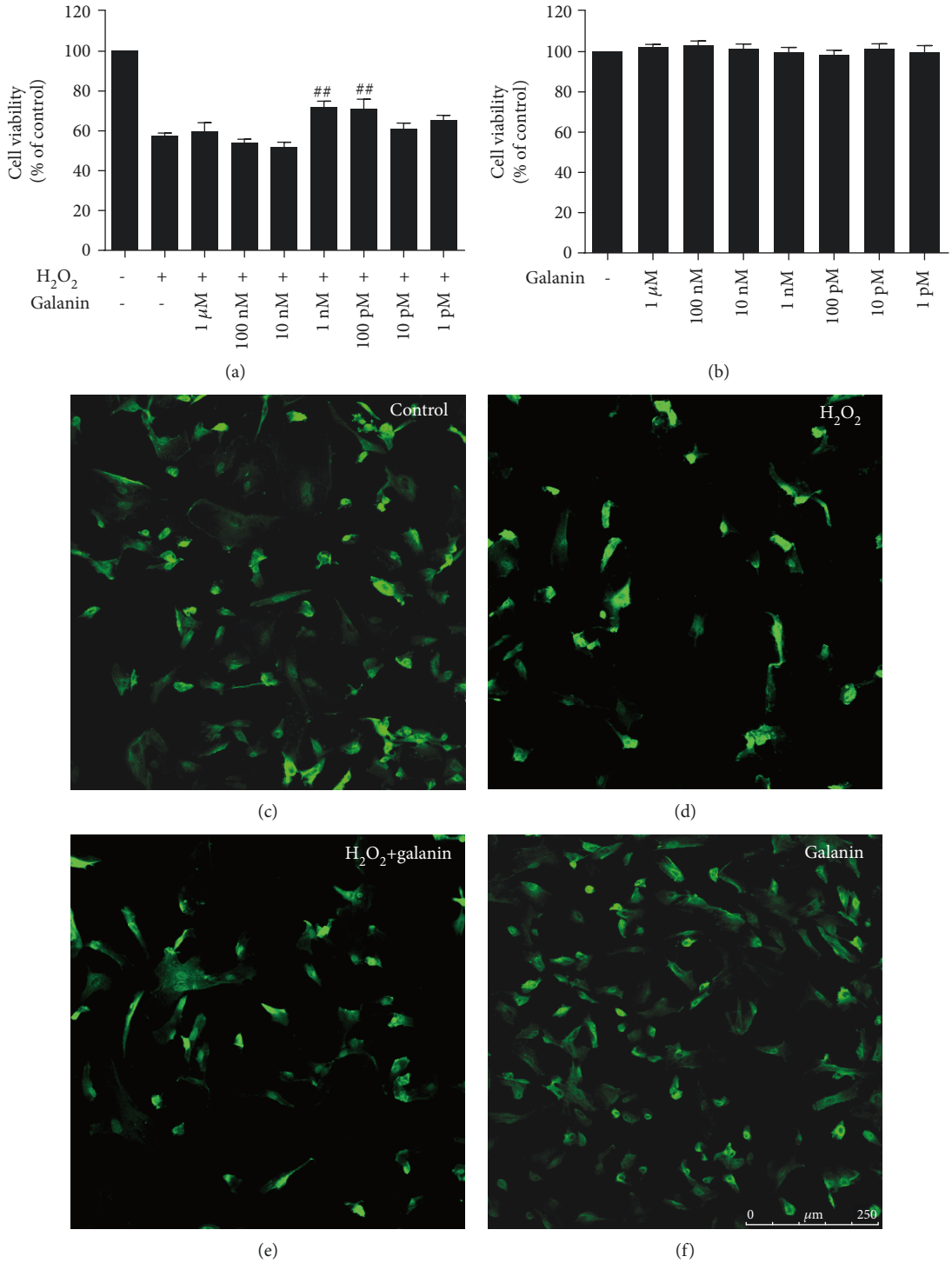

Figure 2: The protective effects of galanin against H_2O_2-induced toxicity in cultured rat cortical astrocytes. (a) Using CCK assay, treatment with 1 nM and 100 pM of galanin showed significantly protective effects against H_2O_2-induced toxicity; (b) treatment with galanin alone did not have any significant effects on the astrocyte viability compared to the control group; (c–f) galanin reversed the cell death induced by H_2O_2. Immunocytochemistry staining of GFAP in different groups: (c) control group, (d) 150 μM H_2O_2 group, (e) 150 μM H_2O_2+1 nM galanin group, and (f) 1 nM galanin group. Data were presented as mean ± SE. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. H_2O_2 group. Bar = 250 μm.

In order to investigate if galanin is able to revert A1 reactive astrocytes back to resting astrocytes, IL-1α, TNFα, and C1q were applied to induce A1 reactive astrocytes [10]. As shown in Figure 6, after the treatment, the body of astrocytes turned hypertrophy. Meanwhile, A1 transcripts such as TNFα, IL-1β, and iNOS in cultured rat cortical astrocytes

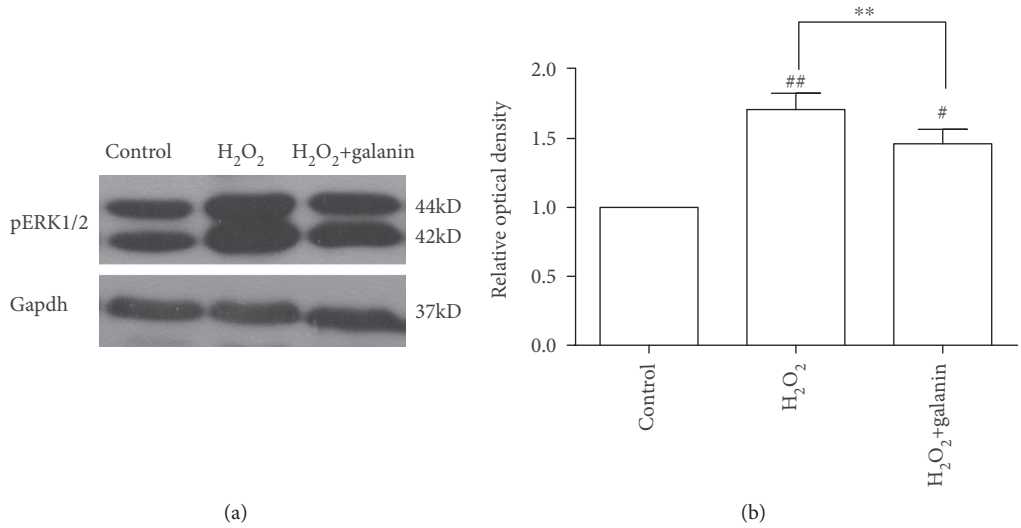

Figure 3: Galanin suppressed the H_2O_2-induced upregulation of the pERK1/2 protein level. (a) Representative blots of pERK1/2 in control, 150 μM H_2O_2, and 150 μM H_2O_2+1 nM galanin groups; (b) ratios of pERK1/2 to Gapdh were calculated and compared. Data were presented as mean ± SE. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the H_2O_2 group.

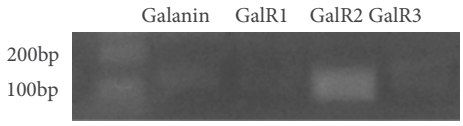

Figure 4: Moderate expression of GalR2 and weak expression of GalR1 and GalR3 in cultured rat cortical astrocytes. The expression of galanin, GalR1, GalR2, and GalR3 was detected by RT-PCR.

were robustly elevated (Figure 7 and Table 3). However, coapplication of galanin had no significant effects on the upregulation of A1 transcripts (Figure 7, Table 3).

4. Discussion

Galanin has been considered as a neurotransmitter/-neuromodulator in the nervous system [23]. Meanwhile, accumulated evidences indicate that galanin also plays a neurotrophic/neuroprotective effect to subsets of neurons in the peripheral and central nervous systems [16]. For example, in both in vivo and in vitro models of injury, more hippocampal neuronal cell death was observed in the galanin knockout mice and less hippocampal neuronal cell death was observed in the galanin-overexpressing transgenic mice, compared with the WT controls [24]. Galanin also inhibits the neurotoxicity induced by amyloid-beta in primary cultured hippocampal neurons from human, rat, and transgenic animal [25, 26].

In the present study, for the first time, we demonstrated that galanin, at concentrations ranging from 100 pM to 1 nM, had significant protective effects on H_2O_2-induced cell death of cultured rat cortical astrocytes. Exogenous H_2O_2 treatment induces reactive oxygen species (ROS) generated intracellularly. When ROS accumulation exceeds cellular capacity, the resulting oxidative stress leads to astrocyte death [27]. H_2O_2 had been shown to enhance the phosphorylation of protein kinase ERK in astrocytes [28]. Although the activation of ERK1/2-MAPK has been considered as a signaling for cell survival, growth, and proliferation, it has also been shown that inhibiting the pERK signal pathway reduced astrocyte death induced by oxidative stress [29]. In the present study, the upregulation of pERK1/2 induced by H_2O_2 was partly reversed by galanin, suggesting that galanin suppressed the H_2O_2-induced toxicity in astrocytes of rats through suppressing the activation of pERK1/2. It should be noted that galanin has also been reported to activate ERK signal in neurons [30]. Thus, the signaling mechanisms of galanin might be different in different cells.

GalR1 and GalR2 are widely expressed in the central nervous system while the expression of GalR3 is limited [31, 32]. It has been demonstrated that the neuroprotective effects of galanin are mediated through its receptors. Thus, galanin has its neuron protection functions through GalR2 [17, 18, 24] or GalR1 [19, 20]. Our study here showed that the GalR2 mRNA expression was moderate while the GalR1 and GalR3 mRNA expression were very low in the cultured rat cortical astrocytes. Moreover, GalR2 agonist AR-M1896 mimicked the protective effects of galanin in the astrocytes. All those results suggest that GalR2 is involved in the protection of cortical astrocytes. This is consistent with previous studies in neurons that GalR2 mediates the protective effects of galanin [18, 33]. Interestingly, galanin had its effect only at concentrations ranging from 100 pM to 1 nM, and AR-M1896 had its effect only at concentrations ranging from 10 pM to 100 nM. The bell-shaped dose responses of galanin has been reported as a critical concentration window in earlier studies [26]. Moreover, we also seen AR-M1896 had broader effective concentration than galanin in the current study. The mechanisms underlying the bell-shaped dose response are still unclear.

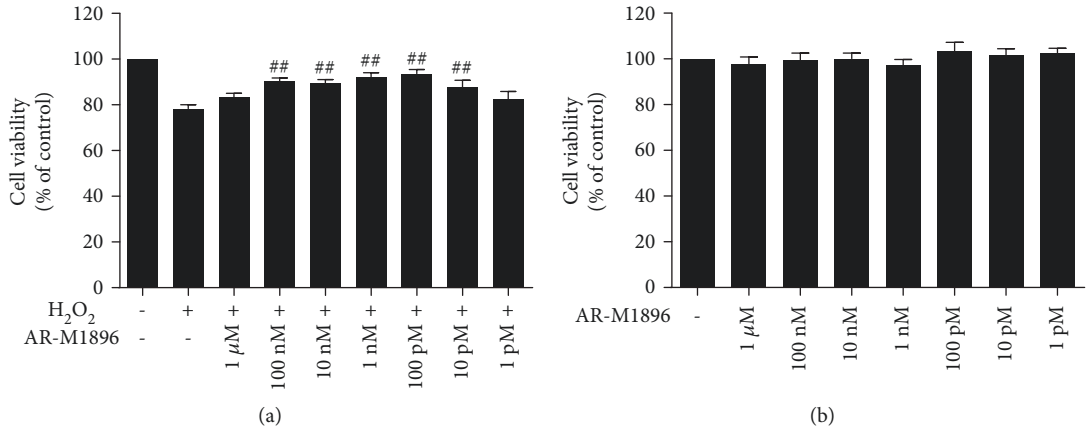

Figure 5: The protective effects of AR-M1896 against H_2O_2-induced toxicity in cultured cortical astrocytes of rats. (a) Using the CCK technique, treatment with 100 nM-10 pM of AR-M1896 showed significant protective effects against H_2O_2-induced toxicity; (b) treatment with AR-M1896 alone did not have any significant effect on the astrocyte viability compared with the control group. Data were presented as mean ± SE. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the H_2O_2 group.

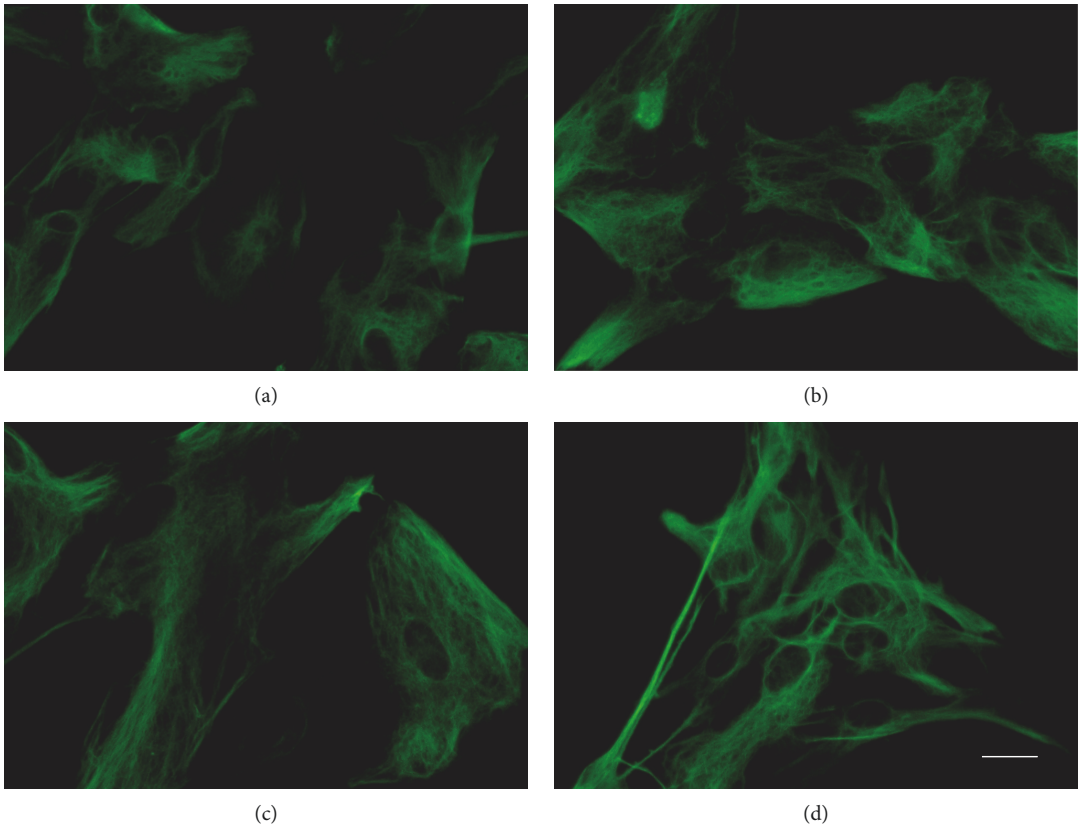

Figure 6: Cultured cortical astrocytes activated by combined application of TNFα, IL-1α, and C1q. The cell body turned hypertrophy. Immunocytochemistry staining of GFAP was detected in astrocytes of different groups: (a) control group, (b) TNFα+IL-1α+C1q group, (c) TNFα+IL-1α+C1q+galanin group, and (d) galanin group. Bar = 20 μm.

One possible explanation is galanin might act on different subtype receptors with different binding affinity. However, it needed to be further investigated.

A recent study shows that astrocytes are transformed to A1 reactive type when treated with a combination of IL-1α, TNFα, and C1q in vitro [10]. A1-type reactive astrocytes are considered to be neurotoxic and contribute to neuron death after acute CNS injury. For example, A1-type astrocytes have been proved taken a role in the neuroinflammation of traumatic spinal cord injury [34]. Block of A1 astrocyte

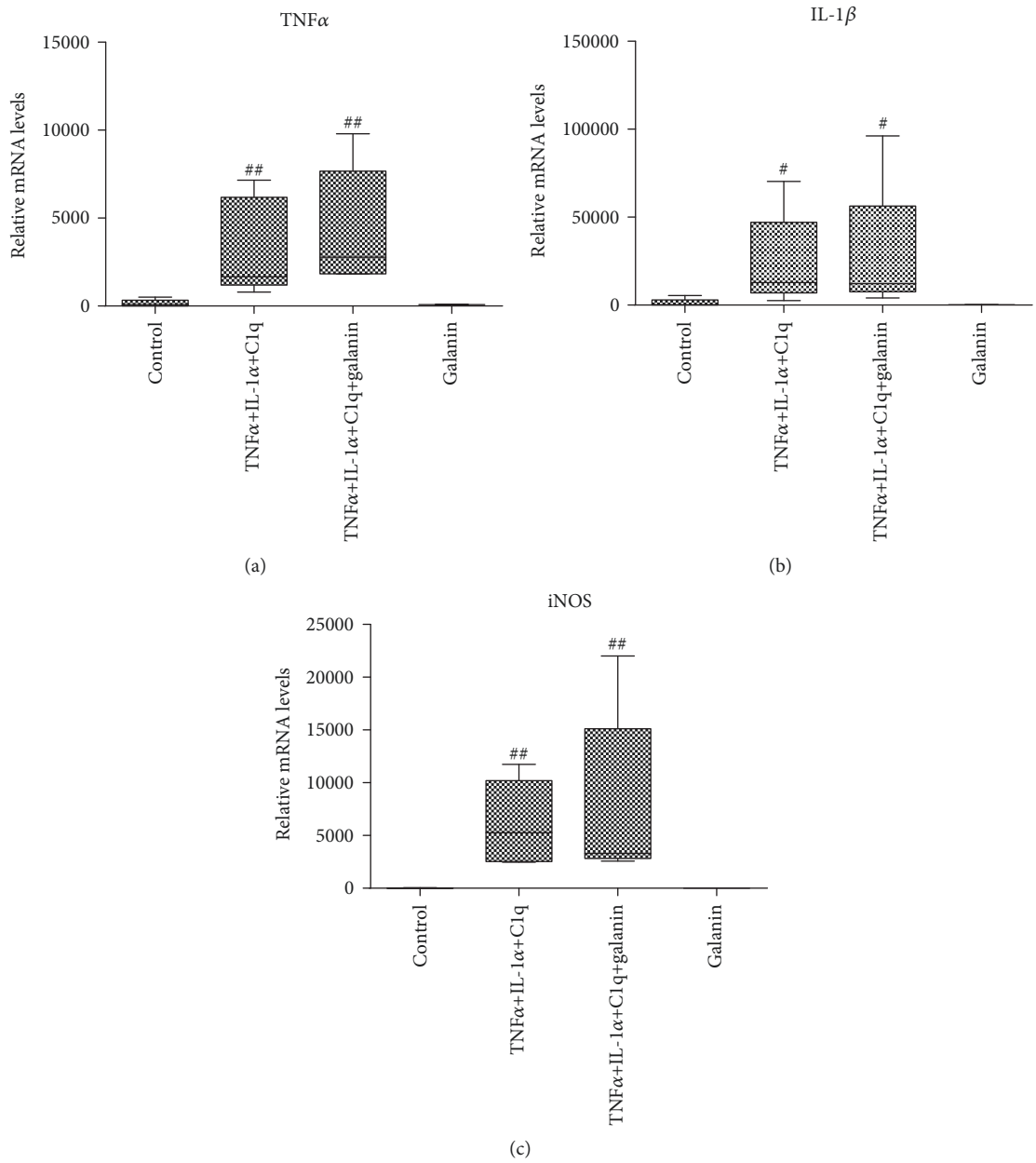

Figure 7: TNFα, IL-1β, and iNOS mRNA of cultured cortical astrocytes elevated by combined application of TNFα, IL-1α, and C1q. Application of galanin did not change A1 phenotype. The relative transcriptional levels of TNFα, IL-1β, and iNOS genes were detected with qPCR. Data were presented as median (interquartile range). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the control group.

Table 3: The relative transcriptional levels of TNFα, IL-1β, and iNOS genes in astrocytes.

	Control	TNFα+IL-1α+C1q	TNFα+IL-1α+C1q+galanin	Galanin
TNFα	85.14 (321.11)	1672.21 (4983.80)	2781.99 (5841.54)	81.13
IL-1β	165.75 (2852.47)	12848.98 (40156.28)	12153.73 (48747.71)	156.90
iNOS	2.56 (28.90)	5250.60 (7667.45)	3292.36 (12294.74)	1.78

conversion by microglia is neuroprotective in models of Alzheimer's disease or Parkinson's disease [35, 36]. Therefore, modulating the harmful effects of A1-type astrocytes could be an important stratagem for the CNS disease treatment. However, galanin had no effect on A1-type transformation of the cultured rat cortical astrocytes in the present study.

5. Conclusion

Galanin protected rat cortical astrocytes from H_2O_2-induced cell death by suppressing the upregulation of pERK1/2, mainly mediated by GalR2.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared that no competing interests exist.

Acknowledgments

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References

[1] M. V. Sofroniew and H. V. Vinters, "Astrocytes: biology and pathology," *Acta Neuropathologica*, vol. 119, no. 1, pp. 7–35, 2010.

[2] M. V. Sofroniew, "Astrocyte barriers to neurotoxic inflammation," *Nature Reviews Neuroscience*, vol. 16, no. 5, pp. 249–263, 2015.

[3] C. Murphy-Royal, J. Dupuis, L. Groc, and S. H. R. Oliet, "Astroglial glutamate transporters in the brain: regulating neurotransmitter homeostasis and synaptic transmission," *Journal of Neuroscience Research*, vol. 95, no. 11, pp. 2140–2151, 2017.

[4] S. Mahmoud, M. Gharagozloo, C. Simard, and D. Gris, "Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release," *Cells*, vol. 8, no. 2, p. 184, 2019.

[5] D. C. Patel, B. P. Tewari, L. Chaunsali, and H. Sontheimer, "Neuron–glia interactions in the pathophysiology of epilepsy," *Nature Reviews Neuroscience*, vol. 20, no. 5, pp. 282–297, 2019.

[6] F. Chan, N. Z. Lax, C. M. Voss et al., "The role of astrocytes in seizure generation: insights from a novel in vitro seizure model based on mitochondrial dysfunction," *Brain*, vol. 142, no. 2, pp. 391–411, 2019.

[7] O. Shandra, A. R. Winemiller, B. P. Heithoff et al., "Repetitive diffuse mild traumatic brain injury causes an atypical astrocyte response and spontaneous recurrent seizures," *The Journal of Neuroscience*, vol. 39, no. 10, pp. 1944–1963, 2019.

[8] M. Santello, N. Toni, and A. Volterra, "Astrocyte function from information processing to cognition and cognitive impairment," *Nature Neuroscience*, vol. 22, no. 2, pp. 154–166, 2019.

[9] J. L. Zamanian, L. Xu, L. C. Foo et al., "Genomic analysis of reactive astrogliosis," *The Journal of Neuroscience*, vol. 32, no. 18, pp. 6391–6410, 2012.

[10] S. A. Liddelow, K. A. Guttenplan, L. E. Clarke et al., "Neurotoxic reactive astrocytes are induced by activated microglia," *Nature*, vol. 541, no. 7638, pp. 481–487, 2017.

[11] K. Tatemoto, A. Rökaeus, H. Jörnvall, T. J. McDonald, and V. Mutt, "Galanin - a novel biologically active peptide from porcine intestine," *FEBS Letters*, vol. 164, no. 1, pp. 124–128, 1983.

[12] T. Melander, T. Hokfelt, and A. Rokaeus, "Distribution of galanin like immunoreactivity in the rat central nervous system," *Journal of Comparative Neurology*, vol. 248, no. 4, pp. 475–517, 1986.

[13] J. Šípková, I. Kramáriková, S. Hynie, and V. Klenerová, "The galanin and galanin receptor subtypes, its regulatory role in the biological and pathological functions," *Physiological Research*, vol. 66, no. 5, pp. 729–740, 2017.

[14] Z. Q. Xu, T. J. Shi, and T. Hokfelt, "Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and -R2 receptors," *The Journal of Comparative Neurology*, vol. 392, no. 2, pp. 227–251, 1998.

[15] T. Hökfelt, S. Barde, Z. Q. D. Xu et al., "Neuropeptide and small transmitter coexistence: fundamental studies and relevance to mental illness," *Frontiers in Neural Circuits*, vol. 12, p. 106, 2018.

[16] S. A. Hobson, A. Bacon, C. R. Elliot-Hunt et al., "Galanin acts as a trophic factor to the central and peripheral nervous systems," in *Galanin*, vol. 102 of Experientia supplementum, pp. 25–38, Springer, Basel, Switzerland, 2010.

[17] C. R. Elliott-Hunt, R. J. P. Pope, P. Vanderplank, and D. Wynick, "Activation of the galanin receptor 2 (GalR2) protects the hippocampus from neuronal damage," *Journal of Neurochemistry*, vol. 100, no. 3, pp. 780–789, 2007.

[18] C. R. Elliott-Hunt, F. E. Holmes, D. M. Hartley, S. Perez, E. J. Mufson, and D. Wynick, "Endogenous galanin protects mouse hippocampal neurons against amyloid toxicity in vitro via activation of galanin receptor-2," *Journal of Alzheimer's Disease*, vol. 25, no. 3, pp. 455–462, 2011.

[19] K. Webling, J. L. Groves-Chapman, J. Runesson et al., "Pharmacological stimulation of GAL_1R but not GAL_2R attenuates kainic acid-induced neuronal cell death in the rat hippocampus," *Neuropeptides*, vol. 58, pp. 83–92, 2016.

[20] Y. Li, Z. Mei, S. Liu et al., "Galanin protects from caspase-8/12-initiated neuronal apoptosis in the ischemic mouse brain via GalR1," *Aging and Disease*, vol. 8, no. 1, pp. 85–100, 2017.

[21] J. Priller, C. A. Haas, M. Reddington, and G. W. Kreutzberg, "Cultured astrocytes express functional receptors for galanin," *Glia*, vol. 24, no. 3, pp. 323–328, 1998.

[22] H.-X. Liu, P. Brumovsky, R. Schmidt et al., "Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: selective actions via GalR1 and GalR2 receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 17, pp. 9960–9964, 2001.

[23] R. Lang, A. L. Gundlach, F. E. Holmes et al., "Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity," *Pharmacological Reviews*, vol. 67, no. 1, pp. 118–175, 2015.

[24] C. R. Elliott-Hunt, B. Marsh, A. Bacon, R. Pope, P. Vanderplank, and D. Wynick, "Galanin acts as a neuroprotective factor to the hippocampus," *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 101, no. 14, pp. 5105–5110, 2004.

[25] Y. Cheng and L. C. Yu, "Galanin protects amyloid-β-induced neurotoxicity on primary cultured hippocampal neurons of rats," *Journal of Alzheimer's Disease*, vol. 20, no. 4, pp. 1143–1157, 2010.

[26] J. Cui, Q. Chen, X. Yue et al., "Galanin protects against intracellular amyloid toxicity in human primary neurons," *Journal of Alzheimer's Disease*, vol. 19, no. 2, pp. 529–544, 2010.

[27] J. A. Dowell and J. A. Johnson, "Mechanisms of Nrf2 protection in astrocytes as identified by quantitative proteomics and siRNA screening," *PLoS One*, vol. 8, no. 7, article e70163, 2013.

[28] J. I. Ito, Y. Nagayasu, T. Ogawa, H. Okihara, and M. Michikawa, "Biochemical properties in membrane of rat astrocytes under oxidative stress," *Brain Research*, vol. 1615, pp. 1–11, 2015.

[29] A. Elmann, A. Telerman, S. Mordechay et al., "3,5,4′-Trihydroxy-6,7,3′-trimethoxyflavone protects astrocytes against oxidative stress via interference with cell signaling and by reducing the levels of intracellular reactive oxygen species," *Neurochemistry International*, vol. 78, pp. 67–75, 2014.

[30] J. J. Hawes, R. Narasimhaiah, and M. R. Picciotto, "Galanin and galanin-like peptide modulate neurite outgrowth via protein kinase C-mediated activation of extracellular signal-related kinase," *European Journal of Neuroscience*, vol. 23, no. 11, pp. 2937–2946, 2006.

[31] D. O'Donnell, S. Ahmad, C. Wahlestedt, and P. Walker, "Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: distinct distribution from GALR1," *Journal of Comparative Neurology*, vol. 409, no. 3, pp. 469–481, 1999.

[32] S. M. Waters and J. E. Krause, "Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues," *Neuroscience*, vol. 95, no. 1, pp. 265–271, 2000.

[33] S. Pirondi, M. Fernandez, R. Schmidt, T. Hokfelt, L. Giardino, and L. Calza, "The galanin-R2 agonist AR-M1896 reduces glutamate toxicity in primary neural hippocampal cells," *Journal of Neurochemistry*, vol. 95, no. 3, pp. 821–833, 2005.

[34] W. Liu, Y. Wang, F. Gong et al., "Exosomes derived from bone mesenchymal stem cells repair traumatic spinal cord injury by suppressing the activation of A1 neurotoxic reactive astrocytes," *Journal of Neurotrauma*, vol. 36, no. 3, pp. 469–484, 2019.

[35] X. Xu, A. Zhang, Y. Zhu et al., "MFG-E8 reverses microglial-induced neurotoxic astrocyte (A1) via NF-κB and PI3K-Akt pathways," *Journal of Cellular physiology*, vol. 234, no. 1, pp. 904–914, 2018.

[36] S. P. Yun, T. I. Kam, N. Panicker et al., "Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease," *Nature Medicine*, vol. 24, no. 7, pp. 931–938, 2018.

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Research Article

NPY Receptor 2 Mediates NPY Antidepressant Effect in the mPFC of LPS Rat by Suppressing NLRP3 Signaling Pathway

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Accumulated evidences show that neuroinflammation play a pivotal role in the pathogenesis of depression. Neuropeptide Y (NPY) and its receptors have been demonstrated to have anti-inflammative as well as antidepressant effects. In the present study, the ability of NPY to modulate depressive-like behaviors induced by lipopolysaccharides (LPS) in rats and the receptors and signaling mechanisms involved were investigated. Continuous injection LPS (i.p) for 4 days led to development of depressive-like behaviors in rats, accompanied with M1-type microglia activation and increased levels of IL-1β as well as decreased levels of NPY and Y2R expression in the mPFC selectively. Local injection of NPY into the medial prefrontal cortex (mPFC) ameliorated the depression-like behaviors and suppressed the NLRP3 inflammasome signaling pathway. Y2R agonist PYY (3-36) mimicked and Y2R antagonist BIIE0246 abolished the NPY effects in the mPFC. All these results suggest that NPY and Y2R in the mPFC are involved in the pathophysiology of depression and NPY plays an antidepressant role in the mPFC mainly via Y2R, which suppresses the NLRP3 signaling pathway, in LPS-induced depression model rats.

1. Introduction

Inflammasome activation in the central nervous system (CNS) and cell-mediated immune response are the prominent feature associated with depression symptom, duration, or severity [1–4]. Studies in postmortem samples of depressed individuals who died by suicide demonstrated that both mRNA and protein levels of IL-1β, IL-6, and TNF-α are significantly increased, and anti-inflammatory cytokine IL-10 and IL-4 are significantly decreased in the PFC [5]. Major depression disorder (MDD) with antidepressant-resistant patients is also accompanied with increased concentration of IL-1, IL-6, TNF-α, and acute phase reactants in plasma compared with treatment-responsive patients [6]. Studies from rattus depression model demonstrated similar results as well [7]. Therefore, prevention of inflammatory disturbances has been acknowledged as a potential avenue for treatment of depression

Neuropeptide Y (NPY) is one of the most abundant peptides in the CNS, which exerts its variety of physiological responses via five receptor subtypes, termed Y1R, Y2R, Y4R, Y5R, and Y6R. NPY and its receptors are widely expressed in brain regions regulating depression and stress resilience, such as cortex, hypothalamus, and hippocampus [8, 9]. Y1R and Y2R are the most abundant receptor types in the CNS [10–12]. Clinical studies showed NPY variant rs16139 and Y2R variant rs6857715 are associated with MDD [13, 14]. Moreover, NPY plays anti-inflammatory

actions via Y1/Y2 receptors in the monocytes and granulocytes of the peripheral blood of lipopolysaccharide- (LPS-) induced inflammation rat model [15].

In the present study, we aimed to investigate the ability of NPY to modulate depressive-like behaviors of LPS-treated rats. Moreover, the receptors and signaling mechanisms involved were also investigated.

2. Materials and Methods

2.1. Animals and Housing. The experiments in this article were performed on adult Sprague-Dawley rats (2 months old, weighing 200-220 g, Beijing Vital River Laboratory Animal Technology Co. Ltd, China). All rats were acclimatized for one week prior to experiment and housed three per standard size cage with food and water available unless special instructions. Animal rooms were maintained a temperature of 20-25°C and a constant light/dark cycle (lights on: 7:00-19:00). The study was approved by the Animal Care Committee at Capital Medical University. Animals were divided into two experimental groups: the control (CTL) group was treated with saline; the LPS group was administered with LPS (Escherichia coli 055: B5, No. L-2880, Sigma-Aldrich, St. Louis, MO, USA), freshly dissolved in sterile saline prior to injection, at a dose of 500 μg/kg. Both the CTL and LPS group rats were injected intraperitoneally between 09:00 and 10:00 a.m. for 4 days. The administered dose and the duration of the treatment were based on a pilot experiment in our lab.

2.2. Depressive-Like Behavior Tests

2.2.1. Open-Field Test. Open-Field Test (OFT) was quantified for 5 min in the apparatus consisted of a black square arena (125 × 125 cm) and a 40 cm high opaque black wall. All rats were placed in a testing room 30 min before the test took place in order to allow them to acclimate. Each rat was gently placed in the center of the open-field box. During the test, rat was allowed to explore freely in the open field. The distance of horizontal and vertical activity was videotaped and quantified with NAY-maze. The arena was carefully cleaned after each test.

2.2.2. Sucrose Preference Test. To verify anhedonia, the sucrose preference test (SPT) was carried out as described in our earlier study [16], Briefly, rats were water-deprived for 8 h, then were presented with two preweighed bottles, one contained with 1% sucrose solution, the other contained with tap water. Moreover, the placement of two bottles (left/right) was counterbalanced and interchanged 30 min after the test started. The total time of SPT is 1 h. Sucrose consumption was calculated according to the following formula: sucrose preference = [sucrose intake/(sucrose intake + water intake)] × 100%.

2.3. Stereotactical Injection. Rats were anesthetized with 6% chloral hydrate (6 ml/kg) administrated i.p, then stereotaxically implanted guide cannula (RWD Life Science and Technology, Shenzhen, China) into the bilateral mPFC (the stereotaxic coordinates were −3.2 mm bregma, −0.5 mm lateral, and 4.0 mm below the surface of the skull) according to *The Rat Brain in Stereotactic Coordinates* [17]. The guide cannula were closed by a stylet, then were fixed onto the skull with 3 stainless steel screws and dental cement. After the surgery, rats were allowed a 6-day recovery. To evaluate the effect of NPY and Y2R on depressive-related behaviors within the mPFC, NPY (1 nmol, Bachem, England), PYY (3-36) (Y2R agonist, 1 nmol, Tocris, England), and BIBE0246 (Y2 antagonist, 40 nmol, Tocris, England) were dissolved in 0.9% saline and infused into the bilateral mPFC once after the last injection of LPS. After infusion, the injection tube was left for 5 min. The doses of NPY, PYY (3-36), and BIBE0246 were chosen based on published literatures [18–20]. The experiments were carried out according to the schedule shown in Figure 1.

2.4. Quantitative Real-Time PCR Analysis. Brains were rapidly separated from the skull, and the mPFC and ventral hippocampus were removed under RNase-free conditions and immediately frozen in liquid nitrogen and then stored at −80°C for later RNA and protein extraction. The total RNA of the mPFC and ventral hippocampus was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) following the manufacturer's instructions. RNA concentrations were measured using the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) with 260 nm/280 nm ratios between 1.8 and 2.2. RNA (1 μg) was reverse transcribed into first-strand cDNA by applying the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA) following the manufacturer's instructions.

Quantitative PCR (Q-PCR) was performed according to previous studies [21]. All assays were run in triplicate, and GAPDH was used as the internal control for each sample. The sequences of the primers used in this study are provided in Table 1. Reaction protocol was 2 min at 60°C and 10 min at 95°C, followed by 40 cycle reactions as 15 s of denaturing at 95°C and 1 min of annealing at 60°C. Samples were held at 10°C at the end of each amplification reaction. The expression levels of target mRNAs are based on the △Ct value.

2.5. Western Blot Analysis. Total protein extraction was as described in our previous studies [22]. The information of primary antibody is shown in Table 2. Membranes were washed for three times (10 min × 3) with Tris-buffered saline-Tween (TBST) and incubated with horseradish peroxidase- (HRP-) conjugated secondary antibody (1,5000, Absin, Beijing, China) at room temperature for 1 h then washed three times (10 min×3) with Tris-buffered saline-Tween (TBST). The bands on the membrane were visualized by enhanced chemiluminescence (ImageQuant LAS 500). ImageJ analysis software (NIH, MD, USA) was applied to quantify the signal. Each experiment was repeated three times, and the results were averaged and normalized.

2.6. IL-1β Assay. Frozen mPFC brain tissue samples were weighed and transferred to tubes on ice containing 10 times volume of test buffer supplied by ELISA kit (EK301B2/2, Multi Science, China). All samples were centrifuged at 3000 rpm (rounds per min) for 10 min. Plasma was collected

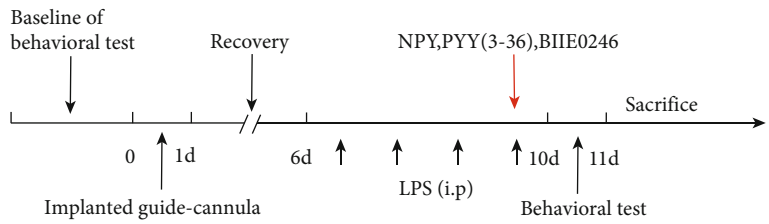

Figure 1: Time schedule for the experiment. LPS was injected for 4 days. Depressive-like behavior tests included OFT and SPT. NPY (1 nmol), Y2R agonist (PYY (3-36), 1 nmol), or Y2R antagonist (BIIE0246, 40 nmol) treatment were locally injected into the mPFC.

Table 1: Sequences of the primers used in Q-PCR.

Primer	Forward	Reverse
GAPDH	GACCACCCAGCCCAGCAAGG	TCCCCAGGCCCCTCCTGTTG
NPY	TGGACTGACCCTCGCTCTAT	TGTCTCAGGGCTGGATCTCT
Y1R	ACACGACTCTTCTTCTGGTGCT	TTACTGTCCCTGATTTTGTCCA
Y2R	GCCTGCCATTCACTCTTACC	CAACGATGTCGGTCCAAAG
Y5R	CTGTCGCCATCCAGTAAGGT	TGGAACGCTTGACTCTCATC
CD11b	CTGGGAGATGTGAATGGAG	ACTGATGCTGGCTACTGATG
Iba-1	TTCCTTCTCTATTACCCCTG	GGTGTTCCTTTTCTTCTCTTGC
MHC-II	AGAGACCATCTGGAGACTTG	CATCTGGGGTGTTGTTGGA
Arginase-1	GGTGGATGCTCACACTGACA	GCAAGCCGATGTACACGATG
Caspase-1	CACGAGACCTGTGCGATCAT	CTTGAGGGAACCACTCGGTC
ASC	TGGCTACTGCAACCAGTGTC	CCAGGCTGGAGCAAAGCTAA
NLRP3	AGCTGCTCTTTGAGCCTGAG	TCTGCTAGGCTCTTTGGTGC
IL-1β	AAATGCCTCGTGCTGTCTGA	GATTCTTCCCCTTGAGGCCC

Table 2: The information of primary antibody.

Antibody	Species	Dilution ratio	Manufacturer
Anti-GAPDH	Mouse source monoclonal antibody	1 : 500	Santa Cruz
Anti-NLRP3	Rabbit source monoclonal antibody	1 : 10000	Abcam
Anti-ASC	Rabbit polyclonal antibody	1 : 500	ImmunoWay
Anti-Caspase-1	Rabbit polyclonal antibody	1 : 500	Proteintech
Anti-IL-1β	Polyclonal goat IgG	1 : 500	R&D systems

and precipitation was abandoned. The standard of IL-1β supplied by ELISA kit was diluted at concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.0312 ng/ml. Levels of IL-1β were measured by ELISA kit based on a standard curve drawn by gradient dilution of the IL-1β standard. The absorbance at 450 nm was measured by using an ELISA plate reader (Multiskan MK3, Thermo Fisher Scientific, USA). Results combined with the measured concentration value and weighing value are expressed as pg/mg.

2.7. Statistical Analysis. The data in this article were presented as mean ± SEM (standard error of measurements). Statistical analysis was analyzed using the SPSS 19.0 software. Student's t-test and one-way ANOVA analysis followed by the LSD multiple comparison tests were selected. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LPS-Induced Depressive-Like Behaviors in Rats. Depressive-like behaviors were assessed on the last day of LPS injection (Figure 1). In OFT, the LPS group rats moved mostly at the edge and rarely at the central area compared with the CTL group in the open-field box (Figure 2(a)). The LPS model rats showed significantly lower horizontal ($P < 0.05$) and vertical scores than the CTL group ($P < 0.05$) (Figures 2(b) and 2(c)). In SPT, the LPS group consumed significantly less sucrose solution than the CTL group ($P < 0.05$) (Figure 2(d)).

3.2. M1-Type Microglia and NLRP3 Inflammasome Signaling Were Activated in the mPFC and Ventral Hippocampus of LPS Model Rats. To assess the phenotype of microglia of LPS-induced inflammation in the CNS, the mRNA expression levels of M1-type microglia markers (CD11b, Iba-1, and

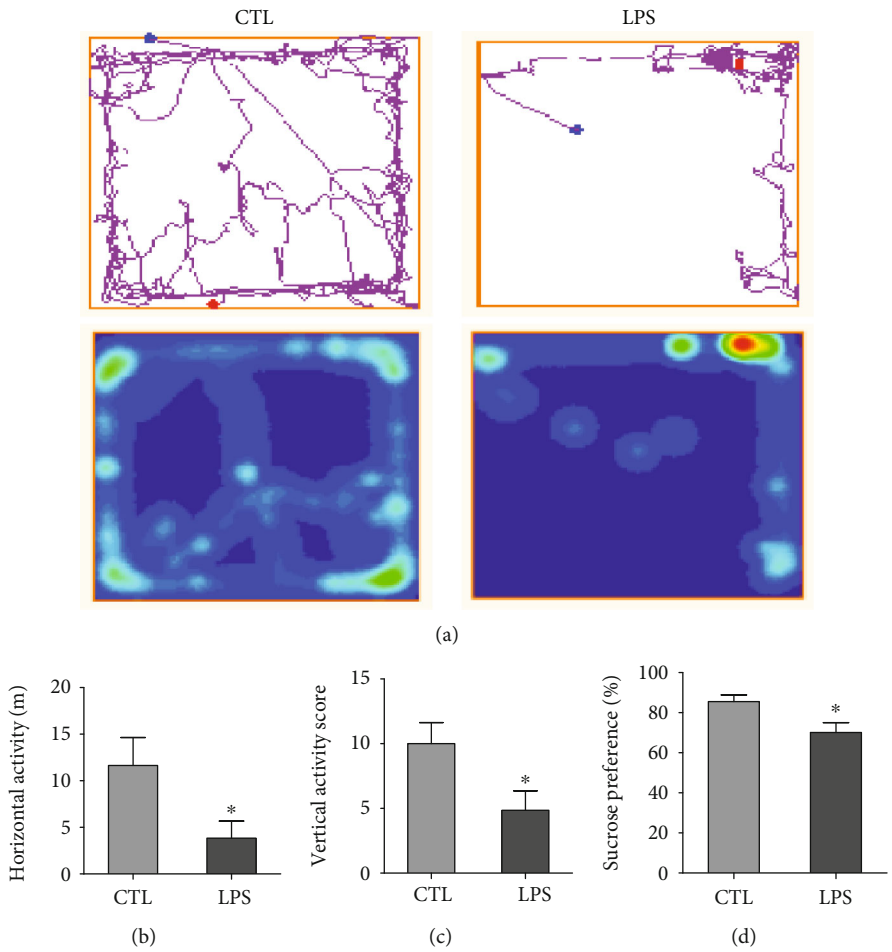

Figure 2: LPS group rats exhibited depressive-like behaviors in SPT and OFT compared with the CTL group. (a) The locomotion track of the CTL and LPS groups. The red area represented the longer residence of the rats, while the green area represents the shorter residence and the blue area represents the least residence of rats. (b, c) LPS group rats showed a lower activity score in OFT compared to the CTL group, horizontal activity score in OFT: $^*P < 0.05$, $t(10) = 2.202$, $P = 0.0479$; vertical activity score in OFT: $^*P < 0.05$, $t(10) = 2.317$, $P = 0.039$. (d) LPS group rats showed a decreased consumption of sucrose in SPT compared to the CTL group, $^*P < 0.05$, $t(10) = 2.611$, $P = 0.0228$. CTL: $n = 6$, LPS: $n = 6$. Values are expressed as mean ± SEM. Independent t-test results are shown in this figure.

Table 3: The mRNA expression of CD11b, Iba-1, MHC-II, Arginase-1, NLRP3, ASC, caspase-1, and IL-1β in the mPFC and ventral hippocampus.

	Markers of M1-type microglia			Markers of M2-type microglia	Markers of NLRP3 pathway			
	CD11b	Iba-1	MHC-II	Arginase-1	NLRP3	ASC	Caspase-1	IL-1β
mPFC								
CTL	0.70 ± 0.08	1.03 ± 0.16	0.73 ± 0.14	1.01 ± 0.07	0.73 ± 0.10	0.70 ± 0.29	0.88 ± 0.10	0.69 ± 0.06
LPS	1.88 ± 0.34	1.58 ± 0.17	1.76 ± 0.36	1.07 ± 0.19	1.14 ± 0.17	1.29 ± 1.71	1.25 ± 0.13	14.54 ± 3.98
Ventral hippocampus								
CTL	0.58 ± 0.11	0.83 ± 0.05	0.68 ± 0.10	0.91 ± 0.07	0.60 ± 0.17	1.24 ± 0.31	0.91 ± 0.10	0.54 ± 0.08
LPS	1.60 ± 0.23	1.47 ± 0.21	1.77 ± 0.35	1.04 ± 0.14	1.07 ± 0.08	1.53 ± 0.06	1.71 ± 0.33	12.51 ± 3.83

MHC-II) and M2-type microglia markers (Arginase-1, CD206, and IL-10) in the mPFC and ventral hippocampus region of rats were analyzed using Q-PCR. The expression of CD11b, Iba-1, and MHC-II in the mPFC was significantly increased in the LPS group rats compared to the CTL group rats (Table 3, Figure 3(a)). Similar results were also seen in the ventral hippocampus (Table 3, Figure 3(c)). Meanwhile, no significant difference in the mRNA expression levels of Arginase-1 was observed in both the mPFC and the ventral hippocampus between two groups (Table 3, Figures 3(a) and 3(c)). The expression level of CD206 and IL-10 was too low to make an appropriate analysis as their CT values were over 35 (data not shown).

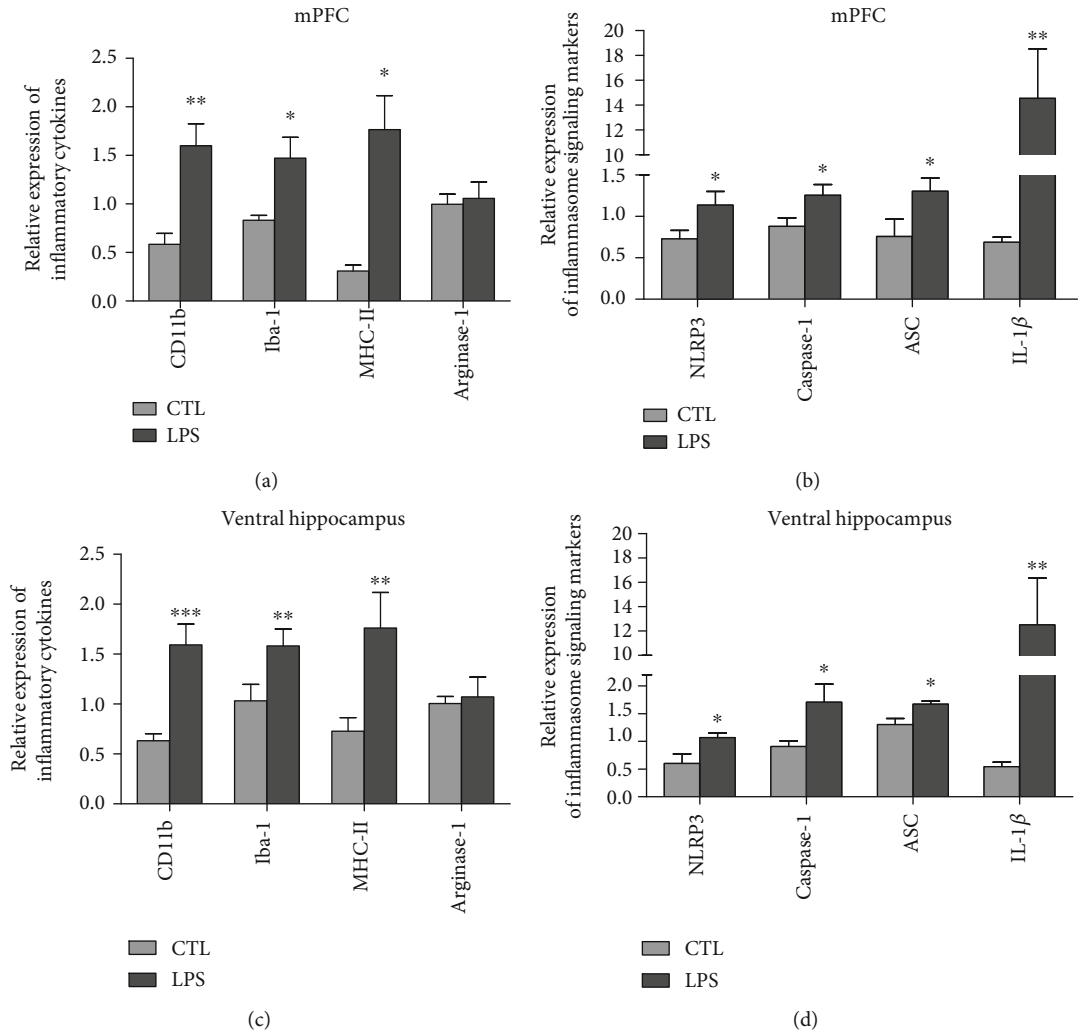

Figure 3: The mRNA expression of M1-type microglia and NLRP3 signaling markers was upregulated in the mPFC and ventral hippocampus of LPS-induced depression rats. (a, c) LPS group rats showed increased mRNA expression of M1-type microglia markers CD11b, Iba-1, and MHC-II compared to CTL rats in the mPFC and ventral hippocampus. In the mPFC: CD11b: $P < 0.01$, $t(10) = 3.348$, $P = 0.0058$; Iba-1: $P < 0.05$, $t(10) = 2.337$, $P = 0.0376$; MHC-II: $P < 0.05$, $t(10) = 2.721$, $P = 0.0186$. In the ventral hippocampus: CD11b: $P < 0.001$, $t(10) = 4.136$, $P = 0.0007$; Iba-1: $P < 0.01$, $t(10) = 2.921$, $P = 0.0091$; MHC-II: $P < 0.01$, $t(10) = 4.123$, $P = 0.0010$. However, there was no significant difference of M2-type microglia marker Arginase-1 between two groups in both the mPFC $P > 0.05$, $t(10) = 0.3215$, $P = 0.7518$ and the ventral hippocampus $P > 0.05$, $t(10) = 0.2578$, $P = 0.6309$ (a, c). (b, d) LPS group rats showed increased mRNA expression of NLRP3 pathway markers NLRP3, caspase-1, ASC, and IL-1β compared to CTL rats. In the mPFC: NLRP3: $P < 0.05$, $t(10) = 2.138$, $P = 0.0473$; casepase-1: $P < 0.05$, $t(10) = 2.276$, $P = 0.0361$; ASC, $P < 0.05$, $t(10) = 2.156$, $P = 0.0421$; IL-1β: $P < 0.01$, $t(10) = 3.678$, $P = 0.009$. In the ventral hippocampus: NLRP3: $P < 0.05$, $t(12) = 2.634$, $P = 0.0272$; caspase-1: $P < 0.05$, $t(10) = 2.322$, $P = 0.0322$; ASC: $P < 0.05$, $t(10) = 2.978$, $P = 0.0139$; IL-1β: $P < 0.01$, $t(10) = 3.306$, $P = 0.0042$. CTL: $n = 6$, LPS: $n = 6$. Values were expressed as mean ± SEM. Data was analyzed by independent t-test.

To determine whether LPS activates M1 phenotype microglia through the NLPR3 signaling pathway, we examined the mRNA expression levels of the NLRP3 pathway markers including NLRP3, caspase-1, ASC, and IL-1β in the mPFC and ventral hippocampus. Thus, NLRP3, caspase-1, ASC, and IL-1β mRNA levels were increased in both the mPFC and the ventral hippocampus from LPS rats compared to control rats (Table 3, Figure 3(b)), indicating an involvement of the NLPR3 signaling pathway.

3.3. NPY and Y2R Transcript Levels Showed a Region-Selective Decrease in LPS Model Rats. Transcript levels of NPY and NPYRS (including Y1R, Y2R, and Y5R) in the mPFC and ventral hippocampus regions from LPS and CTL rats were examined using Q-PCR. The mRNA expression levels of NPY and Y2R were significantly decreased in the mPFC from LPS rats compared with CTL rats (1.10 ± 0.06 vs. 0.82 ± 0.04 and 1.18 ± 0.08 vs. 0.87 ± 0.07, $P < 0.05$, respectively) (Figure 4(a)). However, no significant difference in the expression levels of NPY and Y2R in the ventral hippocampus was seen between two groups (Figure 4(b)). There were no significant differences in the Y1R and Y5R expressions in these two brain regions between the LPS group and the CTL group (Figures 4(a) and 4(b)).

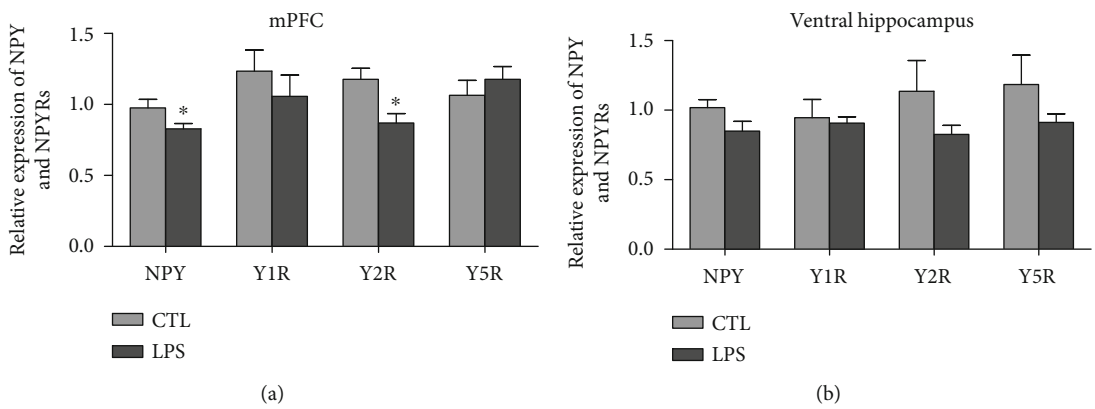

Figure 4: The expression of NPY and Y2R in the mPFC of the LPS group was decreased, while the expression of other NPYRS in the mPFC and ventral hippocampus showed no change. (a) NPY and Y2R showed decreased mRNA expression in the mPFC of the LPS group compared to CTL rats, Y2R: $P < 0.05$, $t(10) = 3.034$, $P = 0.0104$; NPY: $P < 0.05$, $t(10) = 2.195$, $P = 0.0486$. (b) There were no significant differences of Y1R and Y5R expression in the two brain region. CTL: $n = 6$, LPS: $n = 6$. Values were expressed as mean ± SEM. Data were analyzed by independent t-test.

3.4. Injection of NPY or PYY (3-36) into the mPFC Reversed the LPS-Induced Depressive-Like Behaviors. To test if NPY and Y2R play antidepressant roles in the LPS model, NPY (1 nmol), PYY (3-36) (Y2R agonist, 1 nmol), and BIIE0246 (Y2R antagonist, 1 nmol) were injected into the mPFC, and depressive-like behaviors were carried out at the last day of LPS injection (Figure 1). In OFT, NPY and PYY (3-36) reversed the LPS-induced decreases of horizontal and vertical activity score. Thus, the LPS+NPY group showed a significantly higher horizontal activity score and vertical activity score compared to the LPS group (15.83 ± 2.49 vs. 5.65 ± 1.02 and 8.60 ± 1.08 vs. 2.60 ± 0.75, $P < 0.01$, respectively) (Figures 5(b) and 5(c)). The LPS+PYY (3-36) group also showed a significantly higher horizontal activity score and vertical activity score compared to the LPS groups (13.57 ± 0.55 vs. 5.64 ± 1.02, $P < 0.01$ and 12.33 ± 2.39 vs. 2.60 ± 0.75, $P < 0.05$, respectively) (Figures 5(b) and 5(c)). While the LPS+NPY+BIIE0246 group showed a significantly lower horizontal activity score and vertical activity score compared to the LPS+NPY groups (4.26 ± 1.01 vs. 15.83 ± 2.49, $P < 0.001$ and 3.50 ± 0.99 vs. 8.60 ± 1.08, $P < 0.01$, respectively) (Figures 5(b) and 5(c)). In sucrose preference test, the LPS+NPY and LPS+PYY (3-36) group showed a significantly higher sucrose consumption compared to the LPS group (82.80 ± 3.92 vs. $63.17 \pm 6.39\%$ and 81.83 ± 2.48 vs. $63.17 \pm 6.39\%$, $P < 0.05$, respectively) (Figure 5(d)). However, the LPS+NPY+BIIE0246 group only showed a decreased tendency of sucrose consumption (61.83 ± 10.98 vs. $82.80 \pm 3.92\%$, $P = 0.13$) compared to the LPS+NPY group (Figure 5(d)). All those data suggested that application of NPY into the mPFC has antidepressant effect, mainly through Y2R.

3.5. Injection of NPY or PYY (3-36) into the mPFC Reversed the Overactivated NLRP3 Pathway Induced by LPS. To explore whether NPY and Y2R play antidepressant roles by inhibiting the NLRP3 pathway in the mPFC region of LPS rats, we examined the protein expression levels of NLRP3, caspase-1, ASC, and IL-1β after treatment of NPY or PYY (3-36). PYY (3-36) reversed the LPS-induced increase of NLRP3, caspase-1, ASC, and IL-1β levels (Table 4, Figures 6(a)–6(e)). NPY also reversed the LPS-induced increase of caspase-1 and ASC levels while BIIE0246 blocked NPY effects (Table 4, Figures 6(a)–6(e)). Moreover, ELISA results showed that both NPY and PYY (3-36) reversed the LPS-induced upregulation of IL-1β level (Table 4, Figure 6(f)). Meanwhile, BIIE0246 blocked NPY effects on the LPS-induced upregulation of IL-1β in the mPFC (Table 4, Figure 6(f)). All these data suggested that NPY inhibits the NLRP3 pathway via Y2R.

4. Discussion

In the present study, we demonstrated that injection of LPS for 4 days induced depressive-like behaviors. Inflammatory cytokines which collectively polarize the inflammatory M1 phenotype and NLRP3 inflammasome signaling were upregulated accompanied with decreased expression of NPY and Y2R in the mPFC of LPS rats. Moreover, administration of NPY or Y2R agonist PYY (3-36) into the mPFC ameliorated the LPS-induced depressive-like behaviors while inhibited the NLRP3 inflammasome.

Accumulating evidences suggest that central inflammation plays an important role in the development of depression [1–4]. Microglial activation is the principal component of neuroinflammation in the CNS; the function of microglial cells in the pathophysiology of depression has attracted more and more attention. During inflammatory conditions, microglia can be activated with two subtypes, M1 phenotype (proinflammatory) and M2 phenotype (anti-inflammatory) [23, 24]. The predominant subtypes at the injury are M1 phenotype microglia as increased production of CD11b, Iba-1, MHC-II, IL-1β, inducible nitric oxide synthase (iNOS), and the activation of the NLRP3 pathway [25]. Conversely, the activated M2 phenotype is characterized by upregulated anti-inflammatory mediators, such as Arginase-1, CD206, and transforming growth factor-β (TGF-β) [26, 27]. LPS is a powerful immune system activator which has also been

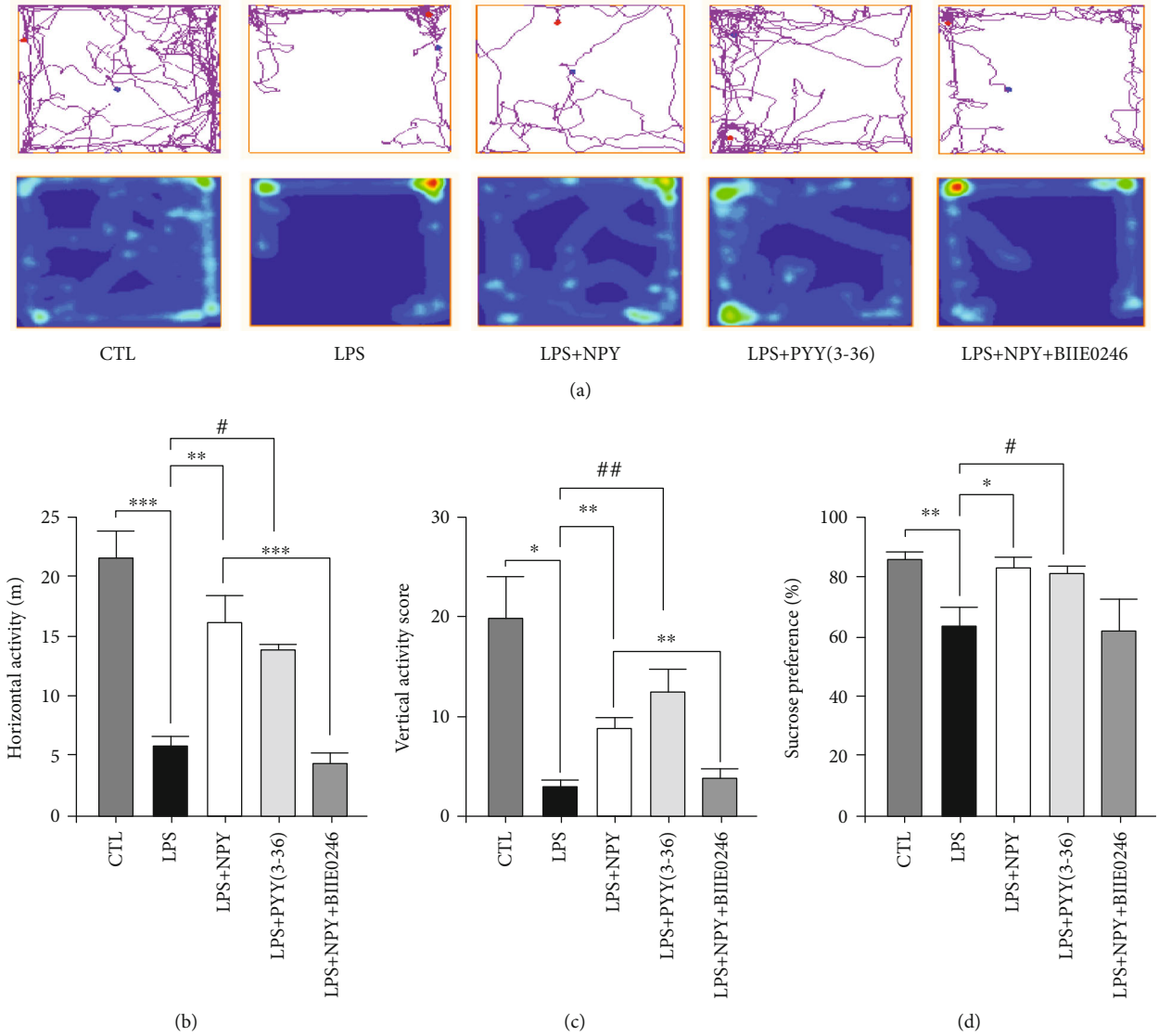

Figure 5: Injection of NPY or PPY (3-36) into the mPFC reversed the LPS-induced depression-like behaviors. (a) The locomotion track of five groups. (b) NPY and PPY (3-36) reversed the LPS-induced decrease of horizontal activity score; BIIE0246 prevented this antidepressant phenomenon of NPY. One-way ANOVA result was $F(4, 31) = 16.931$, $P < 0.001$; (c) NPY or PPY (3-36) treatment prevented the LPS-induced lower vertical activity score; BIIE0246 prevented this effect of NPY. One-way ANOVA result was ($F(4, 31) = 5.6$, $P = 0.002$); (d) NPY or PPY (3-36) treatment prevented the LPS-induced decreased consumption of sucrose in SPT; one-way ANOVA result of SPT was $F(4, 31) = 4.209$, $P = 0.009$. CTL: $n = 9$, LPS: $n = 6$, LPS+NPY: $n = 5$, LPS+PYY (3-36): $n = 6$, LPS+NPY+BIIE0246: $n = 6$; values were expressed as mean ± SEM. Data were analyzed by the LSD multiple comparison tests followed by one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the LPS or LPS+NPY group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the LPS+PYY (3-36) group.

Table 4: The protein expression of the markers of the NLRP3 pathway in the mPFC.

	Western blot				ELISA (pg/mg)
	NLRP3	ASC	Caspase-1	IL-1β	IL-1β
CTL	0.16 ± 0.01	0.39 ± 0.09	0.72 ± 0.03	0.60 ± 0.04	9.30 ± 1.29
LPS	0.28 ± 0.02	0.78 ± 0.06	0.96 ± 0.05	0.91 ± 0.01	20.65 ± 0.65
LPS+NPY	0.17 ± 0.02	0.44 ± 0.03	0.42 ± 0.06	0.77 ± 0.03	16.05 ± 1.63
LPS+PYY (3-36)	0.17 ± 0.01	0.48 ± 0.03	0.51 ± 0.09	0.66 ± 0.05	14.98 ± 2.30
LPS+NPY+BIIE0246	0.28 ± 0.01	0.95 ± 0.08	0.90 ± 0.05	1.31 ± 0.15	24.46 ± 4.42

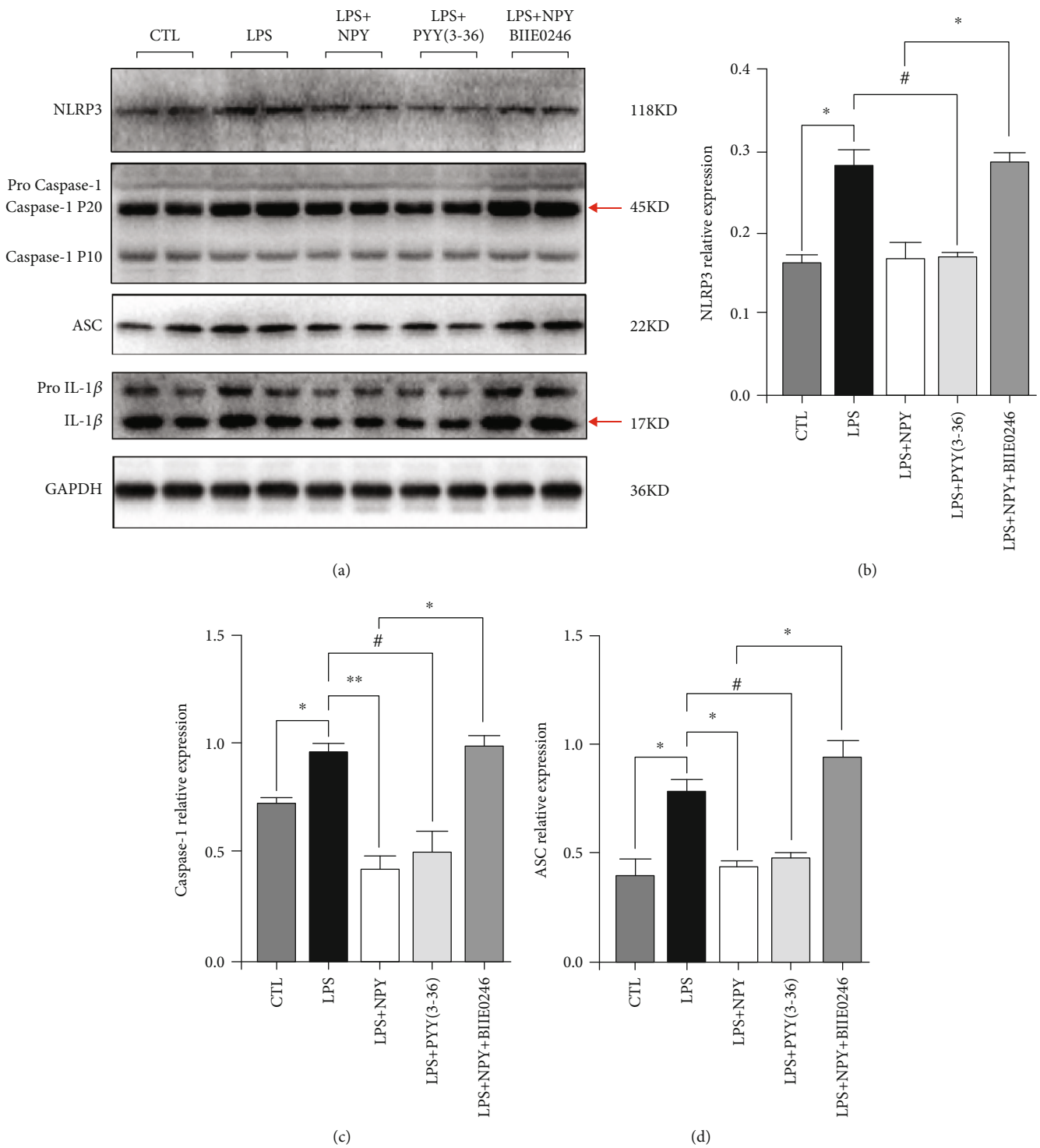

Figure 6: Continued.

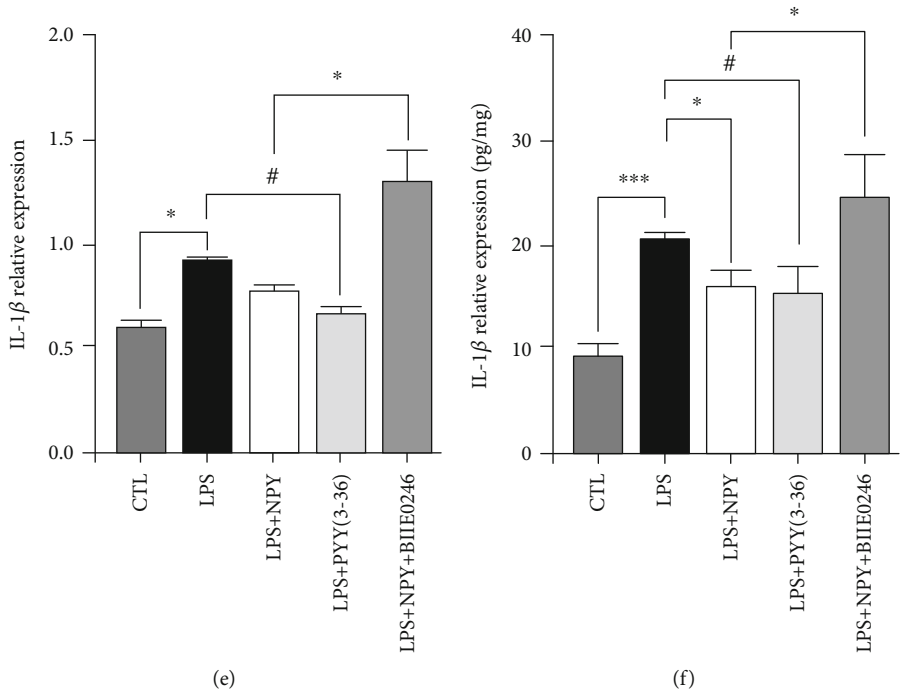

Figure 6: Injection of NPY or PYY (3-36) into the mPFC reversed the LPS-induced overactivation of the NLRP3 pathway. (a) The western blot results of the NLRP3 pathway markers, including NLRP3, caspase-1, ASC, and IL-1β. (b) The LPS group showed significantly increased NLRP3 expression, and PYY (3-36) reversed the increased NLRP3 expression of the LPS group; BIIE0246 significantly increased the NLRP3 expression when compared to the NPY+LPS group; one-way ANOVA result was $F(4, 19) = 47.08$, $P = 0.0001$. (c) The LPS group showed significantly increased caspase-1 expression; NPY and PYY (3-36) reversed the increased caspase-1 expression of the LPS group; BIIE0246 significantly increased caspase-1 expression when compared to the NPY+LPS group. One-way ANOVA result was $F(4, 19) = 55.18$, $P = 0.0001$. (d) The LPS group showed significantly increased ASC expression; NPY and PYY (3-36) reversed the increased ASC expression of the LPS group; BIIE0246 significantly increased ASC expression when compared to the NPY+LPS group. One-way ANOVA result was $F(4, 19) = 43, 68$, $P = 0.0001$. (e) The LPS group showed significantly increased IL-1β expression, and PYY (3-36) reversed the increased IL-1β expression of the LPS group; BIIE0246 significantly increased IL-1β expression when compared to the NPY+LPS group. One-way ANOVA result was $F(4, 19) = 43.74$, $P = 0.0001$. (f) The LPS group rats had significantly increased the expression of IL-1β detected by ELISA compared to the CTL group; NPY and PYY (3-36) reversed the increased IL-1β expression of the LPS group; BIIE0246 significantly increased the IL-1β expression when compared to the NPY+LPS group; one-way ANOVA result was $F(4, 26) = 9.499$, $P = 0.001$. (a–f) CTL: $n = 4$, LPS: $n = 4$, LPS+NPY: $n = 4$, LPS+PYY (3-36): $n = 4$, LPS+NPY+BIIE0246: $n = 4$. (e) CTL: $n = 6$, LPS: $n = 5$, LPS+NPY: $n = 5$, LPS+PYY (3-36): $n = 5$, LPS+NPY+BIIE0246: $n = 6$. Values were expressed as mean ± SEM. Data were analyzed by LSD multiple comparison tests followed by one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the LPS or LPS+NPY group. $^{\#}P < 0.05$, compared with the LPS+PYY (3-36) group.

used to generate immune stress depression model [28, 29]. In the present study, we found that injection of LPS for 4 days induced depressive-like behaviors and increased levels of IL-1β and M1-type microglia activation markers such as CD11b, Iba-1, and MHC-II. Meanwhile, there was no significant difference in the expression of Arginase-1 (M2-type microglia marker) between CTL and LPS rats, suggesting that mainly proinflammatory but not anti-inflammatory was activated in LPS rats. These results were consistent with the earlier studies that the M1 phenotype microglia can be activated by LPS [30–32]. Moreover, our results further verified that neuroinflammation is a contributing factor in the pathophysiology of depression [33–35].

It has been reported that NPY is involved in the pathologic process of depression [36, 37]. The reduction of NPY in the limbic region has been reported in several models of depression including the Flinders Sensitive Line and Fawn Hooded rats as well as chronic mild stress rats [38–40]. Administration of NPY exerts antidepressant-like effects in the olfactory bulbectomized rats and learned helplessness rats [41, 42]. In the present study, we found that the NPY expression was decreased in the mPFC of the inflammation depression rats induced by LPS. Interestingly, while upregulation of inflammatory markers was found in both the mPFC and the ventral hippocampus, downregulation of the NPY expression was only observed in the mPFC, indicating that the regulation of the NPY transcription by LPS is region specific in rats. Moreover, application of NPY in the mPFC reversed the depressive-like behavior in LPS rats, suggesting NPY in the mPFC has antidepressant effect on LPS-induced inflammation depression.

Among five NPY receptor subtypes, Y1R has been shown to mediate NPY-induced antidepressant-like activity in the olfactory bulbectomized rats and the PTSD model rats as well as in the forced swimming test mouse [43–45]. Meanwhile, antagonist of Y5R has antidepressant-like effects in the

CMS rat [46, 47]. In the present study, mRNA expression levels of Y1R, Y2R, and Y5R were measured in both the mPFC and the hippocampus of LPS rats; decreased Y2R expression was only found in the mPFC selectively. The mPFC is one of the dominant brain region that mediated stress response; structural and functional changes of the mPFC have been shown associated with emotional disturbances in human depression patients and rodent depression model [48, 49]. It has been reported that Y2R-like immunoreactivity expresses in the mPFC (both Prl and IL) [11]. Moreover, injection of Y2R agonist into the mPFC had a similar effect of NPY, while Y2R antagonist abolished the antidepressant-like effects of NPY. All those results suggest that Y2R in the mPFC is involved in the pathophysiology of depression and mediates NPY antidepressant effects in LPS rats. However, it has been shown that Y2R antagonist has anti-depressant effect in the olfactory bulbectomized rat [50]. Meanwhile, Y2-/- mice exhibited reduced anxiety-related and depression-like behavior [51]. Therefore, Y2R may play different roles in different depression models and different animals.

NLRP3 inflammasome is a multiple protein complex composed of innate immune sensor NLRP3, ASC, and caspase-1. Activation of NLRP3 complex cleave procaspase-1 to mature caspase-1 and generates bioactive IL-1β and IL-18 [52, 53]. It has been shown that increased IL-1β is only found in PFC after LPS treatment [54]. IL-1β treatment elicits depressive-like behaviors, neuroprogression, and inflammation, and IL-1β antagonists were suggested to play antidepressant roles in several mental disorders [55]. Moreover, it has been reported that NLRP3 signaling plays a key role in microglial activation and inflammation in LPS-induced depression [56]. In agreement with earlier studies, we also showed that expression levels of proinflammatory factors such as NLRP3, caspase-1, ASC, and IL-1β were increased in the mPFC of LPS rats. The LPS-induced upregulations of NLRP3, caspase-1, ASC, and IL-1β were reversed by the application of NPY or PYY (3-36), and the inhibitory effects of NPY were blocked by BIIE0246. The anti-inflammation effect of Y2R agonist has also been reported in endotoxemic animals [57]. Taken together, all those implied that NLRP3 inflammasome-related pathways are involved in antidepressant-like activity of NPY mediated by Y2R in the mPFC of LPS rats. However, the mechanisms underlying the immune-modulating and stress-buffering actions of Y2R contributing to the attenuation of behavioral disturbances caused by peripheral immune challenge are complicated and further studies are required.

5. Conclusion

NPY played an antidepressant role in the mPFC by suppressing the NLRP3 signaling pathway, mainly via Y2R, in the LPS-induced depression model rats.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors have declared that no competing interests exist.

Acknowledgments

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References

[1] H. Eyre and B. T. Baune, "Neuroimmunological effects of physical exercise in depression," *Brain Behavior and Immunity*, vol. 26, no. 2, pp. 251–266, 2012.

[2] M. Iwata, K. T. Ota, and R. S. Duman, "The inflammasome: pathways linking psychological stress, depression, and systemic illnesses," *Brain Behavior and Immunity*, vol. 31, no. 31, pp. 105–114, 2013.

[3] T. Druzhkova, K. Pochigaeva, A. Yakovlev et al., "Acute stress response to a cognitive task in patients with major depressive disorder: potential metabolic and proinflammatory biomarkers," *Metabolic Brain Disease*, vol. 34, no. 2, pp. 621–629, 2019.

[4] A. H. Miller, V. Maletic, and C. L. Raison, "Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression," *Biological Psychiatry*, vol. 65, no. 9, pp. 732–741, 2009.

[5] G. N. Pandey, H. S. Rizavi, H. Zhang, R. Bhaumik, and X. Ren, "Abnormal protein and mRNA expression of inflammatory cytokines in the prefrontal cortex of depressed individuals who died by suicide," *Journal of Psychiatry and Neuroscience*, vol. 43, no. 6, pp. 376–385, 2018.

[6] S. M. O'Brien, P. Scully, P. Fitzgerald, L. V. Scott, and T. G. Dinan, "Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy," *Journal of Psychiatric Research*, vol. 41, no. 3-4, pp. 326–331, 2007.

[7] Y. Liu, Y. Wang, and C. Jiang, "Inflammation: the common pathway of stress-related diseases," *Frontiers in Human Neuroscience*, vol. 11, p. 11, 2017.

[8] T. Hökfelt, C. Broberger, M. Diez et al., "Galanin and NPY, two peptides with multiple putative roles in the nervous system," *Hormone and Metabolic Research*, vol. 31, no. 05, pp. 330–334, 1999.

[9] F. Reichmann and P. Holzer, "Neuropeptide Y: a stressful review," *Neuropeptides*, vol. 55, no. 2, pp. 99–109, 2016.

[10] J. Kopp, Z. Q. Xu, X. Zhang et al., "Expression of the neuropeptide Y Y1 receptor in the CNS of rat and of wild-type and Y1 receptor knock-out mice. Focus on immunohistochemical localization," *Neuroscience*, vol. 111, no. 3, pp. 443–532, 2002.

[11] D. Stanić, P. Brumovsky, S. Fetissov, S. Shuster, H. Herzog, and T. Hökfelt, "Characterization of neuropeptide Y2 receptor protein expression in the mouse brain. I. Distribution in cell bodies and nerve terminals," *The Journal of Comparative Neurology*, vol. 499, no. 3, pp. 357–390, 2006.

[12] A. Santos-Carvalho, C. A. Aveleira, F. Elvas, A. F. Ambrósio, and C. Cavadas, "Neuropeptide Y receptors Y_1 and Y_2 are

present in neurons and glial cells in rat retinal cells in culture," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 1, pp. 429–443, 2013.

[13] Y. Wang, Y. Yang, L. Hui et al., "A neuropeptide Y variant (rs16139) associated with major depressive disorder in replicate samples from Chinese Han population," *PLoS One*, vol. 8, no. 2, article e57042, 2013.

[14] J. Treutlein, J. Strohmaier, J. Frank et al., "Association between neuropeptide Y receptor Y2 promoter variant rs6857715 and major depressive disorder," *Psychiatric Genetics*, vol. 27, no. 1, pp. 34–37, 2017.

[15] K. Mitić, S. Stanojević, N. Kuštrimović, V. Vujić, and M. Dimitrijević, "Neuropeptide Y modulates functions of inflammatory cells in the rat: distinct role for Y1, Y2 and Y5 receptors," *Peptides*, vol. 32, no. 8, pp. 1626–1633, 2011.

[16] P. Wang, H. Li, S. Barde et al., "Depression-like behavior in rat: involvement of galanin receptor subtype 1 in the ventral periaqueductal gray," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 32, pp. E4726–E4735, 2016.

[17] G. Paxinos and C. Watson, *The Rat Brain in Stereotactic Coordinates*, Academic Press, San Diego, CA, USA, 1998.

[18] N. R. Kotagale, N. P. Paliwal, M. M. Aglawe, M. J. Umekar, and B. G. Taksande, "Possible involvement of neuropeptide Y Y1 receptors in antidepressant like effect of agmatine in rats," *Peptides*, vol. 47, no. 47, pp. 7–11, 2013.

[19] G. K. Mittapalli and E. Roberts, "Ligands of the neuropeptide Y Y2 receptor," *Bioorganic & Medicinal Chemistry Letters*, vol. 24, no. 2, pp. 430–441, 2014.

[20] J. C. Morales-Medina, Y. Dumont, C. E. Benoit et al., "Role of neuropeptide Y Y_1 and Y_2 receptors on behavioral despair in a rat model of depression with co-morbid anxiety," *Neuropharmacology*, vol. 62, no. 1, pp. 200–208, 2012.

[21] H. Li, T. Wang, C. Shi et al., "Inhibition of GALR1 in PFC alleviates depressive-like behaviors in postpartum depression rat model by upregulating CREB-BNDF and 5-HT levels," *Frontiers in Psychiatry*, vol. 9, p. 10, 2018.

[22] Y. Li, Z. Mei, S. Liu et al., "Galanin protects from caspase-8/12-initiated neuronal apoptosis in the ischemic mouse brain via GalR1," *Aging and Disease*, vol. 8, no. 1, pp. 85–100, 2017.

[23] X. Hu, R. K. Leak, Y. Shi et al., "Microglial and macrophage polarization—new prospects for brain repair," *Nature Reviews Neurology*, vol. 11, no. 1, pp. 56–64, 2015.

[24] G. Liu and H. Yang, "Modulation of macrophage activation and programming in immunity," *Journal of Cellular Physiology*, vol. 228, no. 3, pp. 502–512, 2013.

[25] S. David and A. Kroner, "Repertoire of microglial and macrophage responses after spinal cord injury," *Nature Reviews Neuroscience*, vol. 12, no. 7, pp. 388–399, 2011.

[26] K. Saijo and C. K. Glass, "Microglial cell origin and phenotypes in health and disease," *Nature Reviews Immunology*, vol. 11, no. 11, pp. 775–787, 2011.

[27] A. Mantovani, S. K. Biswas, M. R. Galdiero, A. Sica, and M. Locati, "Macrophage plasticity and polarization in tissue repair and remodelling," *The Journal of Pathology*, vol. 229, no. 2, pp. 176–185, 2013.

[28] M. Adzic, J. Djordjevic, M. Mitic, Z. Brkic, I. Lukic, and M. Radojcic, "The contribution of hypothalamic neuroendocrine, neuroplastic and neuroinflammatory processes to lipopolysaccharide-induced depressive-like behaviour in female and male rats: involvement of glucocorticoid receptor and C/EBP-β," *Behavioural Brain Research*, vol. 291, pp. 130–139, 2015.

[29] S. Biesmans, L. J. R. Matthews, J. A. Bouwknecht et al., "Systematic analysis of the cytokine and anhedonia response to peripheral lipopolysaccharide administration in rats," *BioMed Research International*, vol. 2016, Article ID 9085273, 14 pages, 2016.

[30] B. A. Durafourt, C. S. Moore, D. A. Zammit et al., "Comparison of polarization properties of human adult microglia and blood-derived macrophages," *Glia*, vol. 60, no. 5, pp. 717–727, 2012.

[31] C. Cunha, C. Gomes, A. R. Vaz, and D. Brites, "Exploring new inflammatory biomarkers and pathways during LPS-induced M1 polarization," *Mediators of Inflammation*, vol. 2016, Article ID 6986175, 17 pages, 2016.

[32] I. Berkiks, S. Boulbaroud, L. M. Garcia-Segura et al., "Thymelaea lythroides extract attenuates microglial activation and depressive-like behavior in LPS-induced inflammation in adult male rats," *Biomedicine & Pharmacotherapy*, vol. 99, pp. 655–663, 2018.

[33] C. E. Millett, B. E. Phillips, and E. F. H. Saunders, "The sex-specific effects of LPS on depressive-like behavior and oxidative stress in the hippocampus of the mouse," *Neuroscience*, vol. 399, pp. 77–88, 2019.

[34] M. Li, C. Li, H. Yu et al., "Lentivirus-mediated interleukin-1β (IL-1β) knock-down in the hippocampus alleviates lipopolysaccharide (LPS)-induced memory deficits and anxiety- and depression-like behaviors in mice," *Journal of Neuroinflammation*, vol. 14, no. 1, p. 190, 2017.

[35] R. Yirmiya, "Endotoxin produces a depressive-like episode in rats," *Brain Research*, vol. 711, no. 1-2, pp. 163–174, 1996.

[36] J. C. Morales-Medina, Y. Dumont, and R. Quirion, "A possible role of neuropeptide Y in depression and stress," *Brain Research*, vol. 1314, no. 2, pp. 194–205, 2010.

[37] A. Thorsell and A. A. Mathé, "Neuropeptide Y in alcohol addiction and affective disorders," *Frontiers in Endocrinology*, vol. 8, p. 13, 2017.

[38] L. Caberlotto, P. Jimenez, D. H. Overstreet, Y. L. Hurd, A. A. Mathé, and K. Fuxe, "Alterations in neuropeptide Y levels and Y1 binding sites in the Flinders Sensitive Line rats, a genetic animal model of depression," *Neuroscience Letters*, vol. 265, no. 3, pp. 191–194, 1999.

[39] A. A. Mathé, P. A. Jimenez, E. Theodorsson, and C. Stenfors, "Neuropeptide Y, neurokinin A and neurotensin in brain regions of Fawn Hooded "depressed", Wistar, and Sprague Dawley rats. Effects of electroconvulsive stimuli," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 22, no. 3, pp. 529–546, 1998.

[40] V. Sergeyev, S. Fetissov, A. A. Mathé et al., "Neuropeptide expression in rats exposed to chronic mild stresses," *Psychopharmacology*, vol. 178, no. 2-3, pp. 115–124, 2005.

[41] C. Song, B. Earley, and B. E. Leonard, "The effects of central administration of neuropeptide Y on behavior, neurotransmitter, and immune functions in the olfactory bulbectomized rat model of depression," *Brain, Behavior, and Immunity*, vol. 10, no. 1, pp. 1–16, 1996.

[42] K. A. Stogner and P. V. Holmes, "Neuropeptide-Y exerts antidepressant-like effects in the forced swim test in rats," *European Journal of Pharmacology*, vol. 387, no. 2, pp. R9–10, 2000.

[43] S. N. Goyal, M. A. Upadhya, D. M. Kokare, S. M. Bhisikar, and N. K. Subhedar, "Neuropeptide Y modulates the antidepressant activity of imipramine in olfactory bulbectomized rats:

involvement of NPY Y1 receptors," *Brain Research*, vol. 1266, no. 8, pp. 45–53, 2009.

[44] L. I. Serova, M. Laukova, L. G. Alaluf, L. Pucillo, and E. L. Sabban, "Intranasal neuropeptide Y reverses anxiety and depressive-like behavior impaired by single prolonged stress PTSD model," *European Neuropsychopharmacology*, vol. 24, no. 1, pp. 142–147, 2014.

[45] J. P. Redrobe, Y. Dumont, A. Fournier, and R. Quirion, "The neuropeptide Y (NPY) Y1 receptor subtype mediates NPY-induced antidepressant-like activity in the mouse forced swimming test," *Neuropsychopharmacology*, vol. 26, no. 5, pp. 615–624, 2002.

[46] M. W. Walker, T. D. Wolinsky, V. Jubian et al., "The novel neuropeptide Y Y_5 receptor antagonist Lu AA33810 [N-[[*trans*-4-[(4,5-dihydro[1]benzothiepino[5,4-*d*]thiazol-2-yl) amino]cyclohexyl]methyl]-methanesulfonamide] exerts anxiolytic- and antidepressant-like effects in rat models of stress sensitivity," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 328, no. 3, pp. 900–911, 2009.

[47] H. Domin, B. Szewczyk, B. Pochwat, M. Woźniak, and M. Śmiałowska, "Antidepressant-like activity of the neuropeptide Y Y5 receptor antagonist Lu AA33810: behavioral, molecular, and immunohistochemical evidence," *Psychopharmacology*, vol. 234, no. 4, pp. 631–645, 2017.

[48] J. S. Seo, J. Wei, L. Qin, Y. Kim, Z. Yan, and P. Greengard, "Cellular and molecular basis for stress-induced depression," *Molecular Psychiatry*, vol. 22, no. 10, pp. 1440–1447, 2017.

[49] L. M. Shin, S. P. Orr, M. A. Carson et al., "Regional cerebral blood flow in the amygdala and medial prefrontalcortex during traumatic imagery in male and female Vietnam veterans with PTSD," *Archives of General Psychiatry*, vol. 61, no. 2, pp. 168–176, 2004.

[50] J. C. Morales-Medina, Y. Dumont, P. Bonaventure, and R. Quirion, "Chronic administration of the Y2 receptor antagonist, JNJ-31020028, induced anti-depressant like-behaviors in olfactory bulbectomized rat," *Neuropeptides*, vol. 46, no. 6, pp. 329–334, 2012.

[51] A. Tschenett, N. Singewald, M. Carli et al., "Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors," *European Journal of Neuroscience*, vol. 18, no. 1, pp. 143–148, 2003.

[52] B. K. Davis, H. Wen, and J. P.-Y. Ting, "The inflammasome NLRs in immunity, inflammation, and associated diseases," *Annual Review of Immunology*, vol. 29, no. 1, pp. 707–735, 2011.

[53] R. V. H. de Carvalho, W. A. Andrade, D. S. Lima-Junior et al., "*Leishmania* lipophosphoglycan triggers caspase-11 and the non-canonical activation of the NLRP3 inflammasome," *Cell Reports*, vol. 26, no. 2, pp. 429–437.e5, 2019.

[54] C. S. Custódio, B. S. F. Mello, R. C. Cordeiro et al., "Time course of the effects of lipopolysaccharide on prepulse inhibition and brain nitrite content in mice," *European Journal of Pharmacology*, vol. 713, no. 1-3, pp. 31–38, 2013.

[55] M. Maes, C. Song, and R. Yirmiya, "Targeting IL-1 in depression," *Expert Opinion on Therapeutic Targets*, vol. 16, no. 11, pp. 1097–1112, 2012.

[56] J. C. O'Connor, C. Andre, Y. Wang et al., "Interferon-γ and tumor necrosis factor-α mediate the upregulation of indoleamine 2,3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus Calmette-Guerin," *The Journal of Neuroscience*, vol. 29, no. 13, pp. 4200–4209, 2009.

[57] J. Stadler, T. P. le, P. Haas, and H. Nave, "Distinct effects of NPY13-36, a specific NPY Y2 agonist, in a model of rodent endotoxemia on leukocyte subsets and cytokine levels," *Annals of Anatomy*, vol. 193, no. 6, pp. 486–493, 2011.

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Review Article

The Association between Depression and Type 1 Diabetes Mellitus: Inflammatory Cytokines as Ferrymen in between?

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The depression incidence is much higher in patients with diabetes mellitus (DM), and the majority of these cases remain underdiagnosed. Type 1 diabetes mellitus (T1D) is now widely thought to be an organ-specific autoimmune disease. As a chronic autoimmune condition, T1D is characterized by T cell-mediated selective loss of insulin-producing β-cells. The age of onset of T1D is earlier than T2D, and T1D patients have an increased vulnerability to depression due to its diagnosis and treatment burden occurring in a period when the individuals are young. The literature has suggested that inflammatory cytokines play a wide role in both diseases. In this review, the mechanisms behind the initiation and propagation of the autoimmune response in T1D and depression are analyzed, and the contribution of cytokines to both conditions is discussed. This review outlines the immunological mechanism of T1D and depression, with a particular emphasis on the role of tumor necrosis factor-α (TNF-α), IL-1β, and interferon-γ (IFN-γ) cytokines and their signaling pathways. The purpose of this review is to highlight the possible pathways of the cytokines shared by these two diseases via deciphering their cytokine cascades. They may provide a basic groundwork for future study of the possible mechanism that links these two diseases and to develop new compounds that target the same pathway but can conquer two diseases.

1. Depression and Diabetes Mellitus

Studies have implicated an increased prevalence of depressive disorders comorbid with anxiety in patients with diabetes mellitus [1]. Depression has an adverse effect in DM and not only significantly increases the risk for other complications but also a predictor for an earlier incidence of complications [2].

Although the cooccurrence of depression in DM could be attributed to a variety of factors, such as the psychological impact of the disease, a potential common genetic susceptibility and cerebrovascular insufficiency that results from neuroimmunological and neuroendocrinical pathways may be present, as well as microvascular brain lesions due to DM [3]. However, the underlying mechanisms concerning the high cooccurrence between these two diseases are not fully profiled yet. Inflammatory cytokines have been found to contribute to the development of depression in both medically healthy and medically ill individuals, including DM [4, 5]. Therefore, we outlined the involved cytokines in both diseases and their possible regulation pathways individually in this review and provided a general idea as to whether circulating cytokines bridge the pathogenesis of these two diseases. Through this summary, we may map out a pathway for the future studies of these two diseases using some common pathways shared by them for disease initiation and

progression. We also evaluated the evidence that associates rheumatoid arthritis with depression, assessing the immune and molecular responses to inflammation.

2. Depression, Inflammation, and Cytokines

2.1. Systemic Inflammation and CNS Depression. Depression is one of the leading causes of disability worldwide, and its incidence is expected to increase. According to the WHO, depression will be the leading cause of disability globally around the year 2030. Although extensive research has been invested into the mechanisms of this disease, the rate of depression is still rising, especially in industrialized countries. The cooccurrence of immune-mediated inflammatory diseases with depression has been well acknowledged in the literature [6]. Persistent low-grade inflammation in peripheral tissues is one of the known predisposing factors in major depression. The overlap timeframes of peripheral and CNS inflammatory responses under immunological conditions raise the possibility of shared pathophysiological mechanisms [6]. Chronic inflammation exacerbates the sickness and depression-like behaviors in patients, which indicates the possible negative effects of inflammatory cytokines on monoaminergic neurotransmission, neurotrophic factors, and measures of synaptic plasticity [7]. Meanwhile, emerging evidence has suggested a strong association between depression and inflammatory processes, and clinical studies have implicated antidepressant treatment effects being boosted with anti-inflammatory agents, both as add-on treatment and as monotherapy [8]. All of the evidence so far has suggested that depression may facilitate inflammatory reactions and cytokine alterations and that inflammation could promote the development of depression. The possible intertwining interactions between them may form a bidirectional loop. Changes in cytokines could be one of the key factors that predispose one to behavioral changes by affecting neurotransmitters, ion channels, and receptors. Therefore, peripheral inflammation may be a key component that closely collaborates with neurotransmitter dysregulation in CNS and adversely affects brain function, leading to psychiatric disorders, including depression.

2.2. Cytokines and CNS Depression. The previous well-established abnormality theory of depression in monoamine systems has been challenged so far, and some studies of functional neuroimaging revealed evidence supporting the relationship between structural/functional anomalies in the brain and a parallel increase of circulating inflammation markers [9]. A handful of previous studies have shown that increases in circulating inflammatory cytokines, including IL-1, TNF-α, and IFN-γ, have been validated in depression [4]. Subjects with depression also demonstrated exacerbated inflammatory responses to stressful impact via upregulation of cytokine levels in the CSF [10], which suggests that circulating cytokines and depression affect each other in a bidirectional way. The changes of those above cytokines in T1D may be the potential culprits that are responsible for the development of depression in DM patients.

2.3. TNF-α. As one of key players of innate immunity, TNF-α is also a physiological regulator of homeostatic cell proliferation, differentiation, and programmed cell death in the CNS [11]. There is emerging evidence implying that TNF-α-producing macrophages play a key role not only in the neurodevelopment but also in the pathophysiology of various neuropsychiatric conditions, including depression [12]. In a recent clinical study, the role of TNF-α in the development of depression and anxiety in a systemic inflammatory disease, systemic lupus erythematosus (SLE), was explored. The results demonstrated that sera TNF-α levels are increased in SLE patients with mood and anxiety disorders. In SLE, TNF-α levels in the sera of SLE patients were independently associated with mood disorders that developed in the patients [13]. These findings suggest the presence of TNF-α and other cytokines may be the immunological basis for depression in SLE. Preclinical study has shown that the deletion of TNF-α is associated with antidepressant-like effects in behavioral tests in mice in comparison with wild-type mice [14], which further implicates TNF-α as having an important role in the development of depression.

2.4. IFN-γ. As a crucial player in innate and adaptive immune responses for many autoimmune diseases, including TID, IFN-γ has recently been recognized to play an important role in stressor-related psychological pathology [15]. Along with other cytokines, IFN-γ levels in the serum of depressed patients were found to be significantly increased compared to their counterparts in a control group in a previous clinical study [16]. In line with the above study, an elevated plasma IFN-γ level was observed in another clinical study with adolescent subjects with depression [17]. More importantly, in the latter study, the researchers further demonstrated the IFN-γ level could be regulated to a normal level after patients underwent effective antidepressant treatment and their symptoms were under control [18]. These results suggest that changes of IFN-γ level are closely associated with depression, and regulation of cytokine expression in patients should be one of the therapeutic targets of treatment. Some preclinical studies also had consistent findings that supported the hypothesis that IFN-γ is a crucial mediator in the pathogenesis of depression [19]. Mice infected with an IFN-γ adenovector demonstrated anhedonic-like symptoms [20]. Meanwhile, several studies showed that some commonly used antidepressants that attenuate depressive symptoms antagonize IFN-γ signaling [21, 22].

2.5. IL-1β. Recent reports have verified that there are increases in proinflammatory cytokine IL-1β in both younger and elderly adults with major depression [23]. Moreover, these studies also provided evidence that the serum IL-1β level of patients is strongly correlated with the severity of their depression and the duration of the current depressive episode. In a preclinical study, increases of IL-1β in serum were associated with acute stress [23]. In addition, the IL-1β level in the CSF of acutely depressed and unmedicated patients was found to be higher than in the control group [24]. Meanwhile, some studies indicated that antidepressant treatment significantly decreased the expression of IL-1β

levels in depression patients. In animal models, IL-1β treatment could effectively cause depressive-like behaviors but treatment with IL-1RA resulted in antidepressant-like effects [25]. In neurodegenerative diseases, such as Alzheimer's disease (AD), IL-1β is also actively responsible for the development of depressive symptoms [26]. IL-1β plasma levels are also significantly associated with cooccurring depressive symptoms in temporal lobe epilepsy [27]. These findings imply that the IL-1β signaling pathway may be one of the common pathways shared by depression and other systemic diseases.

3. Autoimmune Type 1 Diabetes Mellitus and Cytokines

Diabetes mellitus (DM) is a chronic progressive metabolic disorder characterized by hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism, mainly due to defects in insulin secretion, insulin action, or both [28]. DM is one of the oldest recognized diseases, first reported 3000 years ago by the ancient Egyptians [29]. Diabetes is a global public health problem and is now emerging as a pandemic [30]. Diabetes-related complications include cardiovascular disease, nephropathy, neuropathy, retinopathy, and lower-extremity amputation [31, 32]. It results in a significant increase in morbidity and mortality, placing heavy economic burdens on families and health care systems. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades. The World Health Organization (WHO) has estimated that in 2013, approximately 346 million adults were living with diabetes worldwide, compared to 108 million in 1980, and this number is predicted to have almost doubled by 2030 [33].

There are two major forms of diabetes mellitus: type 1 and type 2 diabetes (T1D and T2D). T1D, also known as juvenile or insulin-dependent diabetes mellitus (IDDM), is typically caused by an absolute deficiency of insulin secretion. T2D, previously known as adult or noninsulin-dependent diabetes mellitus (NIDDM), is the result of a progressive deficiency of insulin secretion on the background of resistance to the action of insulin in both peripheral tissues (e.g., muscle and adipose tissues) and β-cells [34].

T1D accounts for 5–10% of the total cases of diabetes worldwide [34]. The disease can occur at any age but is often observed in adolescence and early adulthood. T1D is a chronic autoimmune condition that is characterized by T cell-mediated selective loss of insulin-producing β-cells in the islets of Langerhans of the pancreas. Both genetic and environmental factors contribute to precipitating the disease, and the outcomes of the pathological process depend on multiple interrelated factors. The majority of cases (70–90%) are type 1A diabetes, which is attributable to an autoimmune-mediated destruction of beta cells, while type 1B diabetes (idiopathic) represents a small minority of cases whose specific pathogenesis remains unclear [35]. Although T2D has become increasingly prevalent in children and adolescents, T1D continues to be the most common type of diabetes in this population group, [36]. The understanding of the disease has evolved over the past decade or so, and the age at symptomatic onset is no longer the limiting factor [35]. Children and adolescents with T1D typically present with a hallmark triad of symptoms (i.e., polydipsia, polyphagia, and polyuria), along with overt hyperglycemia, but to a lesser degree in adults [37]. Patients with T1D require lifelong insulin treatment and are prone to ketoacidosis. Technological innovations in insulin pumps and continuous glucose monitors have positively impacted the quality of life of T1D patients since they require lifelong insulin administration. However, effective prevention or cures for T1D remain elusive. Globally, the quality of diabetes management remains uneven. Therefore, it is particularly important to understand the potential pathogenetic mechanisms, to find biomarkers that could be used to predict the progression of T1D and monitor disease activity, and to improve the effectiveness of therapies.

Along with most other autoimmune disorders, multiple genetic susceptibility loci contribute to T1D susceptibility [38]. In addition, nongenetic or environmental factors may contribute to the risk of T1D, given that the concordance rate for Type 1 diabetes in identical twins is not 100% [39]. T1D, therefore, has been suggested to result from a complex interplay between varying degrees of genetic susceptibility and environmental factors [40].

T1D is characterized by progressive lymphocytic infiltration of the pancreatic islets by cells of the immune system—with central roles of $CD4^+$ and $CD8^+$ T cells as well as macrophages [41]. This lymphocyte infiltration can result in inflammatory infiltrates within islets (insulitis) and destruction of insulin production. In some individuals, this infiltration may be held in check through regulation without overt clinical manifestations. In other cases, it can progress to a destructive immune response where β-cells are selectively killed. In most T1D patients, their β-cell mass is reduced by 70–80% at the time of diagnosis [41].

A variety of mechanisms have been proposed to contribute to β-cell death. β-Cell death is probably caused by direct contact with activated macrophages and T cells and/or exposure to soluble mediators secreted by these cells, including cytokines, nitric oxide (NO), and reactive oxygen species (ROS) [41]. Recent experimental and clinical evidence suggest that inflammatory mediators, such as cytokines, might play an important role in the pathogenesis of T1D in addition to metabolic and hemodynamic changes [42–44]. Cytokines are small-secreted proteins that facilitate the interactions and communication between cells, stimulate the proliferation of antigen-specific effector cells, and mediate the local and systemic inflammation via autocrine, paracrine, or endocrine mechanisms [45]. In this review, we discuss recent research progress in the understanding of the roles of inflammatory mediators (especially the cytokines) in the pathogenesis of T1D, which will provide insights into the molecular basis for the early defects and to further develop targeted therapies to better treat T1D.

4. Pathogenesis of T1D

T1D pathogenesis has been extensively studied using experimental animal models, which have greatly enhanced our

understanding of the possible pathogenic features of the disease. The most commonly used models are the nonobese diabetic (NOD) mouse and the diabetes-prone BioBreeding (DP-BB) rats [46]. Although the precise mechanisms responsible for the initiation and progression of β-cell destruction have not been fully elucidated, it is generally believed that β-cell autoantigens, macrophages, dendritic cells (DC), B lymphocytes, and T lymphocytes are involved in triggering β-cell-specific autoimmunity [47]. The autoimmune reaction towards the β-cell appears to begin in the pancreatic lymph nodes (PLN), the site of islet cell-specific self-antigen presentation [48]. In PLN, T cells that have escaped negative selection in the thymus first meet β-cell antigens presented by dendritic cells. T cells migrate to the islets via the circulation and establish insulitis initially around the islets. Regulatory T cells of different cell surface phenotypes and cytokine secretion profiles may also be involved in modulating this unstable equilibrium [49]. Eventually, the chronic process ends in favor of the β-cell-reactive T cells, which eventually causes sufficient reduction of the β-cell mass to render the patient insulin-dependent [50].

Other than the T-cell-mediated β-cell destruction, there are other ways in which β-cell death might occur. It has been reported that there is increased β-cell sensitivity to cytokine-mediated killing since β-cells are particularly sensitive to the cytokine interleukin-1β (IL-1β) [51]. Endoplasmic reticulum (ER) stress is also a factor that increases the sensitivity of islet β-cells to self-directed cellular destruction [52].

5. Cytokines in T1D

The mechanisms involved in β-cell death in T1D may differ. However, many cells that have been shown to produce cytokines have been proven to participate in β-cell destruction in this autoimmune disease. Insulitis is an inflammatory reaction that leads to the loss of most β-cells after long periods of disease [53]. Several cytokines have been shown to play important roles in developing T1D at the level of both immune responses and targeting β-cells. TNF-α, IL-1β, and IFN-γ are the most likely cytokines acting in synergy during inflammation of pancreatic β-cells, leading to the activation of a final common pathway, such as nuclear factor-κB (NF-κB) and, ultimately, to β-cell destruction. NF-κB can be activated by a variety of stimuli, including TNF-α, IL-1, receptor for advanced glycation end products (RAGE), and Toll-like receptors (TLRs).

6. Inflammatory Cytokines in T1D

6.1. TNF-α. TNF-α is a potent pleiotropic proinflammatory cytokine secreted by innate immune cells such as macrophages and monocytes and by differentiated T cells [54]. The evidence suggests that TNF-α exerts its proinflammatory effects by increasing the production of IL-1β and IL-6, expression of adhesion molecules, proliferation of fibroblasts and procoagulant factors, as well as initiation of cytotoxic, apoptotic, acute-phase responses, and inhibition of apoptosis [55]. TNF-α plays multiple roles in the development and function of the immune system and manipulation of TNF-α and its receptors and has revealed numerous aspects of their functions in autoimmune disease, such as T1D.

Early studies reported that, *in vitro*, a combination of TNF-α with IFN-γ resulted in the death of β-cells via the regulation of intraislet IL-1, NO production, and caspase activation [56, 57]. Targeted overexpression of TNF-α in the pancreatic islets of transgenic NOD mice accelerated the progression of diabetes [58]. Studies conducted in adoptive transfer models have suggested that TNF-α plays a critical role in Th1 and Th2 cells in diabetes induction [59, 60]. In accordance with these findings, anti-TNF-α mAb administration to newly onset T1D NOD mice prevented disease progression by restoring euglycemia, self-tolerance, and normal insulin signaling [44, 61]. However, it has been indicated that TNF-α might play a dual role in T1D. Administration of anti-TNF-α treatment at 4 weeks of age or later contributed to the accelerated progression of T1D, while the systemic administration of TNF-α protected against diabetes [62]. Therefore, in T1D experimental models, TNF-α can serve as a double-edged sword by either promoting or inhibiting inflammatory responses. The involved complex factors may include the stage of disease, the length and timing of TNF expression, and the background genetic susceptibility.

6.2. IFN-γ. IFN-γ is a proinflammatory cytokine that is produced in T cells and natural killer cells [63]. It has been reported that IFN-γ plays a key role in driving the autoimmune pathogenesis of T1D. IFN-γ signal transduction requires activation of the tyrosine kinases JAK1 and JAK2 that lead to the phosphorylation of STAT1, which then dimerizes and translocates to the nucleus, binding the γ-activated sites of diverse genes [64]. However, the precise mechanisms by which it contributes to β-cell autoimmunity and T1D progression are not clear yet.

Experimental studies, which were conducted in NOD mice, have reported that the blockade of IFN-γ function via either specific Abs or IFN-γ receptors prevented T1D progression and reduced its incidence [65, 66]. A recent study reported that the T1D candidate gene protein tyrosine phosphatase, type 2 (PTPN2), modulated IFN-γ signal transduction at the β-cell level and might therefore contribute to the pathogenesis of T1D [67]. Although IFN-γ has been generally believed to be important in autoimmune T1D pathogenesis, there are still questions regarding the role of IFN-γ in T1D, since the development of spontaneous β-cell autoimmunity does not change in NOD mice lacking IFN-γ or IFN-γ receptor expression. By employing an adoptive transfer model, it was shown that IFN-γ played an important role in $CD4^+$ T cell-mediated destruction of β-cells instead of $CD8^+$ T cells [68].

Despite a substantial body of evidence, there are still conflicting results in the literature about the possible role of IFN-γ in T1D. It has been suggested that IFN-γ signaling is dispensable for the development of T1D in NOD mice, and in addition, IFN-γ gene disruption may delay but not prevent the onset of T1D [69]. Therefore, such findings need to be repeated and confirmed by other research groups before reaching a consensus.

6.3. IL-1β. IL-1, a prototypical proinflammatory cytokine, has long been believed to cause β-cell dysfunction and death. There is IL-1 expression early in the insulitis infiltrate, and it may be considered as a circulating biomarker of T1D risk. The principal components of the IL-1 family are IL-1α and IL-1β [70]. IL-1α is located within the plasma membrane, whereas IL-1β is exported out of the cell [70]. IL-1β is an inflammatory cytokine that is produced mainly by blood monocytes and also by macrophages, dendritic cells, and a variety of other cells in the body [71].

Studies have indicated that IL-1β plays a major role in mediating both impaired function and destruction of pancreatic β-cells during the development of autoimmune T1D [72]. Secretion of insulin was inhibited by the treatment of rodent islets with IL-1β, followed by β-cell destruction [73]. In human islets, the results showed that exposure of β-cells to IL-1β or IL-1β plus IFN-γ led to β-cell functional changes similar to those that were observed in prediabetic patients [74]. IL-1β has a preferential inhibitory effect on the first phase of glucose-induced insulin release via reducing the docking and fusion of insulin granules to the β-cell membrane [75]. Based on pharmacological studies, p38 MAPK and c-Jun N-terminal kinase (JNK), which are members of the mitogen-activated protein kinases (MAPKs), are specifically activated in IL-1β–mediated β-cell dysfunction [76]. However, there was moderate or no protection from anti-IL-1 strategies or genetic ablation of IL-1 or IL-1RTI in animal models of T1D [77]. In contrast to this disappointing result, in a recent clinical study, it was reported that IL-1β pathway blockade in T1D causes a reduction in monocyte trafficking [78]. There are a few factors that may cause negative outcomes beyond the obvious possibilities, including the intervention time, the administration dosage, the C-peptide decline, and the efficacy of IL-1 antagonism [79].

7. Intracellular Signaling Pathways Activated by Proinflammatory Cytokines in T1D

Pancreatic β-cells are the targets of an autoimmune assault in T1D, with invasion of the islets by mononuclear cells in insulitis, causing loss of most β-cells after prolonged periods of disease [80]. During the course of insulitis, β-cell death results from direct contact with activated macrophages and T-cells or their secreted soluble mediators. As the main cause of β-cell death at the onset of T1D, apoptosis is a process that is highly regulated, activated, and/or modified by extracellular signals, intracellular ATP levels, phosphorylation cascades, and expression of pro- and antiapoptotic genes. There are transcriptional factors and signaling pathways involved in the progression of β-cell death. Proinflammatory cytokines play a key role in the cytokine-promoted β-cell "decision" to undergo apoptosis [41]. It has been identified in microarray experiments that approximately 700 genes and expressed sequence tags are up- or downregulated in purified rat β-cells or insulin-producing cells after exposure to IL-1β and/or IFN-γ [81, 82]. The transcriptional factors NF-κB and STAT-1 are the main regulators of the pathways triggered by IL-1β, IFN-γ, and TNF-α.

7.1. IL-1β Signaling. The transcription factor nuclear factor kappa B (NF-κB) is known to be the main mediator of IL-1β signaling [83]. Similar to the identified pathway by which IL-1β activates NF-κB in various cell types and experimental models, the signaling pathway in β-cells involves TRNF6, JNK, and IKKs. After being produced and released by macrophages and T cells, IL-1β can bind to its receptor 1 (IL-1R1) on the cell surface of target cells. And then IL-1R1 can recruit IL-1 receptor accessory protein (IL-1RAcP) [84], which allows for combination of the adaptor protein myeloid differentiation factor 88 (MyD88) and the recruitment of IL-1R1-activated kinase 1 (IRAK1) and/or IRAK2 [85]. IRAK proteins are found in a complex with a protein named Tollip prior to recruitment to the receptor. Tollip transiently associates with IL-1RacP during recruitment of the Tollip–IRAK complexes to the activated receptor complex [86]. Then, the recruitment of TNF-receptor-associated factor-6 (TRAF6) to IRAK1 and IRAK2 exerts an activation of inhibitors of NF-κB (IκB) kinase (IKK) via NF-κB-inducing kinase (NIK) [87]. IKK then phosphorylates IκB and triggers the degradation and release of NF-κB from the inhibitory interaction. Additionally, in response to IL-1, there is an activation of phosphatidyl inositol-3 kinase (PI3K) [88]. PI3K activity is required (but not sufficient) for NF-κB activation [88].

There are several target genes that can be regulated by NF-κB, including cytokines, chemokines, cell adhesion molecules, apoptosis regulators, and other transcriptional factors. NF-κB signaling is highly cell type-specific. In β-cells, NF-κB activation exerts a proapoptotic effect, while NF-κB activation promotes cell survival in most cells [89]. Studies suggest that inhibition of NF-κB exerts a protective effect on β-cells against damage *in vivo* and *in vitro* [89]. In primary rat β-cells treated with cytokines, there are a large number of NF-κB-targeted genes that have been identified via DNA microarray technology [82]. This study also reported various genes that are regulated by cytokines after induced NF-κB activation. There is upregulation of genes that are involved in immune responses (e.g., MHC-II-associated invariant chain γ and MHC-I) and stress responses (including CHOP, C/EBPβ and δ, Hsp27, and MnSOD), while there is downregulation of genes that are involved in β-cell function (glucose transporter-2 (Glut-2)), insulin production (Isl-1), insulin processing (PC-1), insulin release (PLD-1, CCKA-receptor), and Ca^{2+} homeostasis (SERCA2, IP 3-kinase) [82]. It is known that iNOS is strongly induced by NF-κB in both rat β-cells and human pancreatic islets [81, 90], which may induce the production of ROS and oxidative stress damage. In addition to NF-κB, IL-1β signaling is also able to activate mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) 1/2 and suppress cytokine signaling-3 (SOCS-3) [91]. MAPKs and SOCS-3-induced signaling pathways are interlinked with NF-κB-regulated pathways. MAPK has been shown to potentiate IL-1β-dependent NF-κB activation and iNOS production, while ERK 1/2 activation contributes to cytokine-induced apoptosis in rat pancreatic β-cells [92]. However, SOCS is believed to provide a negative feedback to affect NF-κB signaling and β-cell death. SOCS-3 demonstrates an inhibition of IL-

1β signaling upstream and thus downregulates all effects of IL-1β. There are a variety of IL-1β-induced proapoptotic genes suppressed by SOCS-3, most of which are NF-κB-dependent [91]. SOCS was also shown to protect rat β-cells against IL-1β- and TNFα-induced cell death [92]. Although there have been advances in identifying NF-κB-regulated genes in β-cells, to which extent the expression is regulated by IL-1β alone has not been well studied. The determination of individual targets of the cytokines will be helpful in better understanding of the role of cytokines in T1D in the future.

7.2. TNF-α Signaling. It has been demonstrated that TNF-α contributes to the activation of NF-κB in pancreatic β-cells [93]. TNF-α binds to and activates the TNF receptor (TNFR1), which is present on the surface of β-cells [94]. Binding of the ligand TNF-α to TNFR1 leads to the activation of TNFR1, which induces the recruitment of TNF receptor-associated death domain (TRADD), TRAF2, and the death domain kinase receptor interacting protein (RIP) [95]. TRAF2 then recruits IKK to TNF-R1 while RIP mediates IKK kinase activation [96]. The activation of NF-κB requires phosphorylation of IκB. The activation of NF-κB by TNF-α exerts a proapoptotic effect on rat pancreatic β-cells [89]. TNF-α signaling leads to MAPKs activation (c-Jun N-terminal kinase JNK, p38, and ERK) in a cell type-specific manner. It has been shown that in rat pancreatic β-cells, JNK and p38, which are induced by the TNF-α, exert inhibitory effects on glucose-stimulated insulin secretion and cause impaired β-cell function [97].

7.3. IFN-γ Signaling. Full biologic function of IFN-γ, the homodimeric cytokine, is mediated by the receptor complex, which consists two species-matched chains, IFN-γR1 and IFN-γR2 [98]. IFN-γR1 is the major ligand-binding subunit, while IFN-γR2 increases the IFN-γR1 affinity for its ligand and plays a minor role in direct ligand binding [99]. Ligand binding leads to receptor oligomerization, with two IFN-γR1 chains bound to one IFN-γ homodimer, and the subsequent recruitment of two IFN-γR2 chains to the complex [100]. It has been known that the ligand binding results in the activation and transphosphorylation of Janus tyrosine kinase 1 and 2 (JAK1 and JAK2), which are linked with IFNγR1 and IFNγR2, respectively [101]. IFN-γ signaling is initiated by JAK2 autophosphorylation, which is followed by JAK1 phosphorylation. Then, the activated JAK1 phosphorylates IFN-γR1, providing a docking site for STAT1. After binding to its receptor site, STAT1 is activated by the phosphorylation and activation of JAK2. Furthermore, STAT-1 has been shown to homodimerize and translocate to the nucleus where it stimulates target gene expression [102]. In a STAT-1$^{-/-}$ NOD mouse model, islets treated with IFN-γ and TNF-α or IFN-γ and IL-1β were shown to be resistant to apoptosis [103]. Consistent with the results above, STAT1 blockade prevented diabetes progression in the mouse model induced by injection of multiple low doses of streptozotocin [104]. In a recent gene expression analysis, STAT-1 was found to regulate 2000 genes in response to cytokine (IL-1β and IFN-γ) exposure in β-cells [105]. STAT-1 is a regulator of IL-1β/IFN-γ-mediated induction of chemokines (e.g., CXCL9, CXCL10, CXCL11, and CCL20) [105]. It has been shown that *in vitro* and *in vivo* there is a reduced production of CXCL10 in response to cytokine exposure [106]. Additionally, STAT-1 is able to downregulate several genes that are specific to the functions of β-cells, such as glucokinase, insulin, Glut2, and prohormone convertases, as well as transcriptional factors involved in the differentiation and maintenance of the β-cell phenotype (e.g., Pdx1, MafA, and Nkx2.2) [107]. Finally, STAT-1 plays a key role in the regulation of genes that mediate intracellular stress and apoptotic pathways. The apoptosis-related genes that are regulated by STAT-1 include Puma, CHOP, Bid, caspase-3, -4, -7, DP5/Hrk, and endoplasmic reticulum stress-transducing genes (XBP1, ATF4) [108]. IFN-γ also plays an important role in inducing IL-1β-mediated iNOS and causing oxidative stress damage. It has been indicated that treatment of a rat insulinoma cell line (RIN-r) with a combination of IL-1β and IFN-γ could induce the mitochondrial apoptotic pathway via an iNOS-dependent manner [109]. In T1D, IFN-γ exerts inflammatory effects via negative feedback regulation of interferon-regulated factor-1 (IRF-1) as well as SOCS-1 and SOCS-3 [109, 110]. Via the STAT-1 regulatory role, IRF-1 exerts an inhibitory effect on chemokine expression in β-cells and leads to T cell infiltration of Langerhans islets [111]. Transgenic expression of SOCS-1 in β-cells was shown to protect β-cells against infiltrating autoreactive T cells and to be able to prevent diabetes development in NOD mice [112, 113]. Taken together, in β-cells, IFN-γ activates the STAT-1 signaling pathway and regulates the key processes, which are essential steps in the loss of β-cell function, stress, and finally death. In addition, IFN-γ leads to an increased sensitivity of β-cells to apoptotic stimuli and intracellular stress via the regulation of a number of genes.

8. Conclusion

During the last decade, a great deal has been learned about the role of cytokines in the autoimmune disease of T1D and depression. In this review, we have summarized the latest evidence regarding the role of cytokines and the signaling pathways involved in T1D (Figure 1) and depression. We outlined the most relevant cytokines in these two diseases to generate an idea that cytokines may be the common pathway shared by these two disorders. In addition, several signaling pathways also regulate these diseases' development through mechanisms that have not been fully investigated yet. Currently, there is uncertainty around this topic, as there have been paradoxical results regarding these cytokines while using similar or even the same models. This suggests that there is a lot more to be learned. The discovery of new cytokines and the regulatory mechanisms underlying these two diseases will help to improve our understanding in this field. Successful translation of findings from animal models to clinical trials, while adopting a mechanistic perspective, may yield new approaches for the management of T1D and depression, reducing their incidence and prevalence, limit pharmacological toxicity, and avoid global immunosuppression.

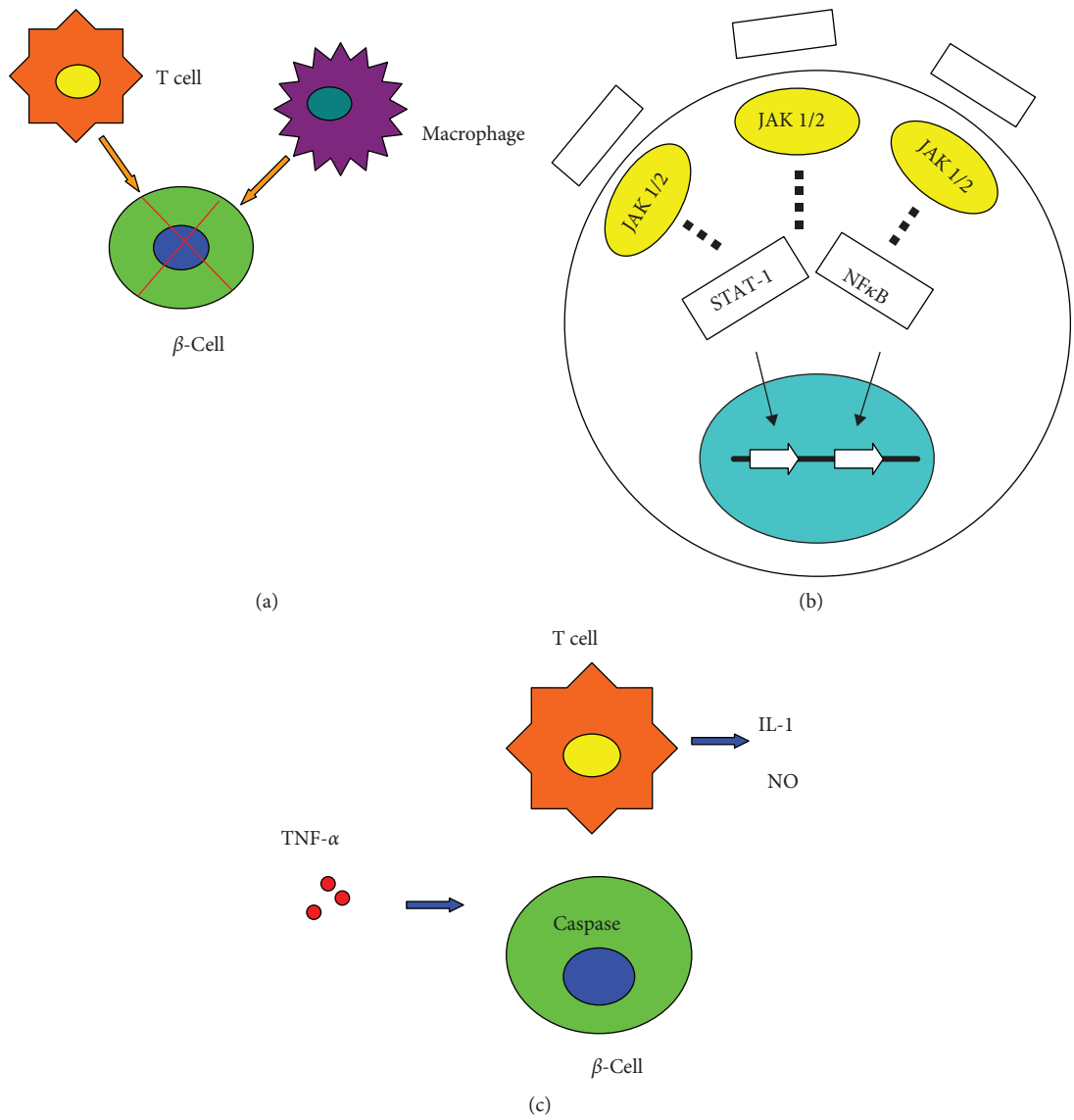

Figure 1

Conflicts of Interest

All authors declare there are no competing interests in this study.

Authors' Contributions

Dr. Kexin Wang drafted the manuscript. Drs. Fangna Li, Yixin Cui, Chunhui Cui, Zhenzhen Cao, Kai Xu, Shuhong Han, and Ping Zhu extensively edited the manuscript. Dr. Yu Sun proposed the idea, designed the outline of the manuscript, and took the responsibility for the integrity of the publication.

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References

[1] M. R. Muscatello, G. M. Troili, G. Pandolfo et al., "Depression, anxiety and anger in patients with type 1 diabetes mellitus," *Recenti Progressi in Medicina*, vol. 108, no. 2, pp. 77–82, 2017.

[2] E. Andreoulakis, T. Hyphantis, D. Kandylis, and A. Iacovides, "Depression in diabetes mellitus: a comprehensive review," *Hippokratia*, vol. 16, no. 3, pp. 205–214, 2012.

[3] S. V. Bădescu, C. Tătaru, L. Kobylinska et al., "The association between diabetes mellitus and depression," *Journal of Medicine and Life*, vol. 9, no. 2, pp. 120–125, 2016.

[4] J. C. Felger and F. E. Lotrich, "Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications," *Neuroscience*, vol. 246, pp. 199–229, 2013.

[5] M. Peyrot, R. R. Rubin, T. Lauritzen et al., "Resistance to insulin therapy among patients and providers: results of the cross-national Diabetes Attitudes, Wishes, and Needs (DAWN) study," *Diabetes Care*, vol. 28, no. 11, pp. 2673–2679, 2005.

[6] A. Inserra, C. A. Mastronardi, G. Rogers, J. Licinio, and M. L. Wong, "Neuroimmunomodulation in Major depressive disorder: focus on caspase 1, inducible nitric oxide synthase, and interferon-gamma," *Molecular Neurobiology*, 2018.

[7] L. Nerurkar, S. Siebert, I. B. McInnes, and J. Cavanagh, "Rheumatoid arthritis and depression: an inflammatory perspective," *The Lancet Psychiatry*, vol. 6, no. 2, pp. 164–173, 2019.

[8] O. Kohler, J. Krogh, O. Mors, and M. Eriksen Benros, "Inflammation in depression and the potential for anti-inflammatory treatment," *Current Neuropharmacology*, vol. 14, no. 7, pp. 732–742, 2016.

[9] B. Bortolato, A. F. Carvalho, J. K. Soczynska, G. I. Perini, and R. S. McIntyre, "The involvement of TNF-α in cognitive dysfunction associated with major depressive disorder: an opportunity for domain specific treatments," *Current Neuropharmacology*, vol. 13, no. 5, pp. 558–576, 2015.

[10] T. W. W. Pace, T. C. Mletzko, O. Alagbe et al., "Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress," *The American Journal of Psychiatry*, vol. 163, no. 9, pp. 1630–1633, 2006.

[11] L. Bernardino, F. Agasse, B. Silva, R. Ferreira, S. Grade, and J. O. Malva, "Tumor necrosis factor-α modulates survival, proliferation, and neuronal differentiation in neonatal subventricular zone cell cultures," *Stem Cells*, vol. 26, no. 9, pp. 2361–2371, 2008.

[12] M. Berthold-Losleben and H. Himmerich, "The TNF-α system: functional aspects in depression, narcolepsy and psychopharmacology," *Current Neuropharmacology*, vol. 6, no. 3, pp. 193–202, 2008.

[13] M. Postal, A. T. Lapa, N. A. Sinicato et al., "Depressive symptoms are associated with tumor necrosis factor alpha in systemic lupus erythematosus," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 5, 2016.

[14] B. B. Simen, C. H. Duman, A. A. Simen, and R. S. Duman, "TNFα signaling in depression and anxiety: behavioral consequences of individual receptor targeting," *Biological Psychiatry*, vol. 59, no. 9, pp. 775–785, 2006.

[15] D. Litteljohn, E. Nelson, and S. Hayley, "IFN-γ differentially modulates memory-related processes under basal and chronic stressor conditions," *Frontiers in Cellular Neuroscience*, vol. 8, p. 391, 2014.

[16] N. M. Simon, K. McNamara, C. W. Chow et al., "A detailed examination of cytokine abnormalities in major depressive disorder," *European Neuropsychopharmacology*, vol. 18, no. 3, pp. 230–233, 2008.

[17] V. Gabbay, R. G. Klein, C. M. Alonso et al., "Immune system dysregulation in adolescent major depressive disorder," *Journal of Affective Disorders*, vol. 115, no. 1-2, pp. 177–182, 2009.

[18] J. Dahl, H. Ormstad, H. C. D. Aass et al., "The plasma levels of various cytokines are increased during ongoing depression and are reduced to normal levels after recovery," *Psychoneuroendocrinology*, vol. 45, pp. 77–86, 2014.

[19] J. C. O'Connor, M. A. Lawson, C. André et al., "Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice," *Molecular Psychiatry*, vol. 14, no. 5, pp. 511–522, 2009.

[20] A. Kwant and B. Sakic, "Behavioral effects of infection with interferon-gamma adenovector," *Behavioural Brain Research*, vol. 151, no. 1-2, pp. 73–82, 2004.

[21] D. Brustolim, R. Ribeiro-dos-Santos, R. E. Kast, E. L. Altschuler, and M. B. P. Soares, "A new chapter opens in anti-inflammatory treatments: the antidepressant bupropion lowers production of tumor necrosis factor-alpha and interferon-gamma in mice," *International Immunopharmacology*, vol. 6, no. 6, pp. 903–907, 2006.

[22] M. Kubera, A. H. Lin, G. Kenis, E. Bosmans, D. van Bockstaele, and M. Maes, "Anti-inflammatory effects of antidepressants through suppression of the interferon-γ/interleukin-10 production ratio," *Journal of Clinical Psychopharmacology*, vol. 21, no. 2, pp. 199–206, 2001.

[23] K. T. Nguyen, T. Deak, S. M. Owens et al., "Exposure to acute stress induces brain interleukin-1β protein in the rat," *The Journal of Neuroscience*, vol. 18, no. 6, pp. 2239–2246, 1998.

[24] J. Levine, Y. Barak, K. N. R. Chengappa, A. Rapoport, M. Rebey, and V. Barak, "Cerebrospinal cytokine levels in patients with acute depression," *Neuropsychobiology*, vol. 40, no. 4, pp. 171–176, 1999.

[25] M. Maes, C. Song, and R. Yirmiya, "Targeting IL-1 in depression," *Expert Opinion on Therapeutic Targets*, vol. 16, no. 11, pp. 1097–1112, 2012.

[26] A. Ng, W. W. Tam, M. W. Zhang et al., "IL-1β, IL-6, TNF- α and CRP in elderly patients with depression or Alzheimer's disease: systematic review and meta-analysis," *Scientific Reports*, vol. 8, no. 1, article 12050, 2018.

[27] É. L. M. Vieira, G. N. M. de Oliveira, J. M. K. Lessa et al., "Interleukin-1β plasma levels are associated with depression in temporal lobe epilepsy," *Epilepsy & Behavior*, vol. 53, pp. 131–134, 2015.

[28] World Health Organization, *Definition, Diagnosis and Classification of Diabete Mellitus and Its Complications*, World Health Organization, 1999.

[29] A. M. Ahmed, "History of diabetes mellitus," *Saudi Medical Journal*, vol. 23, no. 4, pp. 373–378, 2002.

[30] K. M. Venkat Narayan, E. W. Gregg, A. Fagot-Campagna, M. M. Engelgau, and F. Vinicor, "Diabetes — a common, growing, serious, costly, and potentially preventable public health problem," *Diabetes Research and Clinical Practice*, vol. 50, pp. S77–S84, 2000.

[31] S. M. Grundy, I. J. Benjamin, G. L. Burke et al., "Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association," *Circulation*, vol. 100, no. 10, pp. 1134–1146, 1999.

[32] W. T. Cade, "Diabetes-related microvascular and macrovascular diseases in the physical therapy setting," *Physical Therapy*, vol. 88, no. 11, pp. 1322–1335, 2008.

[33] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[34] American Diabetes Association, "2. Classification and diagnosis of diabetes," *Diabetes Care*, vol. 39, Supplement 1, pp. S13–S22, 2015.

[35] M. A. Atkinson, G. S. Eisenbarth, and A. W. Michels, "Type 1 diabetes," *The Lancet*, vol. 383, no. 9911, pp. 69–82, 2014.

[36] D. M. Maahs, N. A. West, J. M. Lawrence, and E. J. Mayer-Davis, "Epidemiology of type 1 diabetes," *Endocrinology and Metabolism Clinics of North America*, vol. 39, no. 3, pp. 481–497, 2010.

[37] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 33, Supplement 1, pp. S62–S69, 2009.

[38] D. V. Serreze and E. H. Leiter, "Genes and cellular requirements for autoimmune diabetes susceptibility in nonobese diabetic mice," *Current Directions in Autoimmunity*, vol. 4, pp. 31–67, 2001.

[39] J. Kaprio, J. Tuomilehto, M. Koskenvuo et al., "Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland," *Diabetologia*, vol. 35, no. 11, pp. 1060–1067, 1992.

[40] O. Vaarala, M. A. Atkinson, and J. Neu, "The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity," *Diabetes*, vol. 57, no. 10, pp. 2555–2562, 2008.

[41] M. Cnop, N. Welsh, J. C. Jonas, A. Jorns, S. Lenzen, and D. L. Eizirik, "Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes: many differences, few similarities," *Diabetes*, vol. 54, Supplement 2, pp. S97–S107, 2005.

[42] J. A. Emamaullee, J. Davis, S. Merani et al., "Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice," *Diabetes*, vol. 58, no. 6, pp. 1302–1311, 2009.

[43] H. E. Thomas, R. Darwiche, J. A. Corbett, and T. W. H. Kay, "Interleukin-1 plus γ-interferon-induced pancreatic β-cell dysfunction is mediated by β-cell nitric oxide production," *Diabetes*, vol. 51, no. 2, pp. 311–316, 2002.

[44] L. F. Lee, B. Xu, S. A. Michie et al., "The role of TNF-α in the pathogenesis of type 1 diabetes in the nonobese diabetic mouse: analysis of dendritic cell maturation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 44, pp. 15995–16000, 2005.

[45] J. M. Zhang and J. An, "Cytokines, inflammation, and pain," *International Anesthesiology Clinics*, vol. 45, no. 2, pp. 27–37, 2007.

[46] A. J. F. King, "The use of animal models in diabetes research," *British Journal of Pharmacology*, vol. 166, no. 3, pp. 877–894, 2012.

[47] J. W. Yoon and H. S. Jun, "Autoimmune destruction of pancreatic β cells," *American Journal of Therapeutics*, vol. 12, no. 6, pp. 580–591, 2005.

[48] P. Höglund, J. Mintern, C. Waltzinger, W. Heath, C. Benoist, and D. Mathis, "Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes," *Journal of Experimental Medicine*, vol. 189, no. 2, pp. 331–339, 1999.

[49] R. Bottino, P. Lemarchand, M. Trucco, and N. Giannoukakis, "Gene- and cell-based therapeutics for type I diabetes mellitus," *Gene Therapy*, vol. 10, no. 10, pp. 875–889, 2003.

[50] M. Thunander, H. Thorgeirsson, C. Törn, C. Petersson, and M. Landin-Olsson, "β-Cell function and metabolic control in latent autoimmune diabetes in adults with early insulin versus conventional treatment: a 3-year follow-up," *European Journal of Endocrinology*, vol. 164, no. 2, pp. 239–245, 2011.

[51] P. Pirot, A. K. Cardozo, and D. L. Eizirik, "Mediators and mechanisms of pancreatic beta-cell death in type 1 diabetes," *Arquivos Brasileiros de Endocrinologia & Metabologia*, vol. 52, no. 2, pp. 156–165, 2008.

[52] S. A. Tersey, Y. Nishiki, A. T. Templin et al., "Islet β-cell endoplasmic reticulum stress precedes the onset of type 1 diabetes in the nonobese diabetic mouse model," *Diabetes*, vol. 61, no. 4, pp. 818–827, 2012.

[53] G. Klöppel, M. Löhr, K. Habich, M. Oberholzer, and P. U. Heitz, "Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited," *Survey and Synthesis of Pathology Research*, vol. 4, no. 2, pp. 110–125, 1985.

[54] J. R. Bradley, "TNF-mediated inflammatory disease," *The Journal of Pathology*, vol. 214, no. 2, pp. 149–160, 2008.

[55] T. Horiuchi, H. Mitoma, S. I. Harashima, H. Tsukamoto, and T. Shimoda, "Transmembrane TNF-α: structure, function and interaction with anti-TNF agents," *Rheumatology*, vol. 49, no. 7, pp. 1215–1228, 2010.

[56] M. D. McKenzie, E. M. Carrington, T. Kaufmann et al., "Proapoptotic BH3-only protein Bid is essential for death receptor–induced apoptosis of pancreatic β-cells," *Diabetes*, vol. 57, no. 5, pp. 1284–1292, 2008.

[57] M. Arnush, A. L. Scarim, M. R. Heitmeier, C. B. Kelly, and J. A. Corbett, "Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes," *The Journal of Immunology*, vol. 160, no. 6, pp. 2684–2691, 1998.

[58] E. A. Green and R. A. Flavell, "The temporal importance of TNFα expression in the development of diabetes," *Immunity*, vol. 12, no. 5, pp. 459–469, 2000.

[59] J. Cantor and K. Haskins, "Effector function of diabetogenic CD4 Th1 T cell clones: a central role for TNF-α," *The Journal of Immunology*, vol. 175, no. 11, pp. 7738–7745, 2005.

[60] J. He and K. Haskins, "Pathogenicity of T helper 2 T-cell clones from T-cell receptor transgenic non-obese diabetic mice is determined by tumour necrosis factor-α," *Immunology*, vol. 123, no. 1, pp. 108–117, 2008.

[61] M. Koulmanda, M. Bhasin, Z. Awdeh et al., "The role of TNF-α in mice with type 1- and 2- diabetes," *PLoS One*, vol. 7, no. 5, article e33254, 2004.

[62] X. D. Yang, R. Tisch, S. M. Singer et al., "Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process," *Journal of Experimental Medicine*, vol. 180, no. 3, pp. 995–1004, 1994.

[63] C. Sano, K. Sato, T. Shimizu, H. Kajitani, H. Kawauchi, and H. Tomioka, "The modulating effects of proinflammatory cytokines interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), and immunoregulating cytokines IL-10 and transforming growth factor-beta (TGF-β), on antimicrobial activity of murine peritoneal macrophages against *Mycobacterium avium-intracellulare* complex," *Clinical & Experimental Immunology*, vol. 115, no. 3, pp. 435–442, 1999.

[64] D. L. Eizirik, F. Moore, D. Flamez, and F. Ortis, "Use of a systems biology approach to understand pancreatic β-cell death in type 1 diabetes," *Biochem Soc Trans*, vol. 36, no. 3, pp. 321–327, 2008.

[65] I. L. Campbell, T. W. Kay, L. Oxbrow, and L. C. Harrison, "Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi

mice," *Journal of Clinical Investigation*, vol. 87, no. 2, pp. 739–742, 1991.

[66] F. Nicoletti, P. Zaccone, R. di Marco et al., "The effects of a nonimmunogenic form of murine soluble interferon-gamma receptor on the development of autoimmune diabetes in the NOD mouse," *Endocrinology*, vol. 137, no. 12, pp. 5567–5575, 1996.

[67] F. Moore, M. L. Colli, M. Cnop et al., "PTPN2, a candidate gene for type 1 diabetes, modulates interferon-γ–induced pancreatic β-cell apoptosis," *Diabetes*, vol. 58, no. 6, pp. 1283–1291, 2009.

[68] Z. Yi, L. Li, A. Garland et al., "IFN-γ receptor deficiency prevents diabetes induction by diabetogenic $CD4^+$, but not $CD8^+$, T cells," *European Journal of Immunology*, vol. 42, no. 8, pp. 2010–2018, 2012.

[69] D. V. Serreze, C. M. Post, H. D. Chapman, E. A. Johnson, B. Lu, and P. B. Rothman, "Interferon-gamma receptor signaling is dispensable in the development of autoimmune type 1 diabetes in NOD mice," *Diabetes*, vol. 49, no. 12, pp. 2007–2011, 2000.

[70] C. A. Dinarello, "The interleukin-1 family: 10 years of discovery," *The FASEB Journal*, vol. 8, no. 15, pp. 1314–1325, 1994.

[71] K. Wang, F. Song, A. Fernandez-Escobar, G. Luo, J. H. Wang, and Y. Sun, "The properties of cytokines in multiple sclerosis: pros and cons," *The American Journal of the Medical Sciences*, vol. 356, no. 6, pp. 552–560, 2018.

[72] T. Mandrup-Poulsen, "The role of interleukin-1 in the pathogenesis of IDDM," *Diabetologia*, vol. 39, no. 9, pp. 1005–1029, 1996.

[73] K. Yamada, N. Takane-Gyotoku, X. Yuan, F. Ichikawa, C. Inada, and K. Nonaka, "Mouse islet cell lysis mediated by interleukin-1-induced Fas," *Diabetologia*, vol. 39, no. 11, pp. 1306–1312, 1996.

[74] K. Hostens, D. Pavlovic, Y. Zambre et al., "Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release," *Journal of Clinical Investigation*, vol. 104, no. 1, pp. 67–72, 1999.

[75] M. Ohara-Imaizumi, A. K. Cardozo, T. Kikuta, D. L. Eizirik, and S. Nagamatsu, "The cytokine interleukin-1β reduces the docking and fusion of insulin granules in pancreatic β-cells, preferentially decreasing the first phase of exocytosis," *Journal of Biological Chemistry*, vol. 279, no. 40, pp. 41271–41274, 2004.

[76] C. D. Major and B. A. Wolf, "Interleukin-1β stimulation of c-Jun NH_2-terminal kinase activity in insulin-secreting cells: evidence for cytoplasmic restriction," *Diabetes*, vol. 50, no. 12, pp. 2721–2728, 2001.

[77] D. L. Eizirik and T. Mandrup-Poulsen, "A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis," *Diabetologia*, vol. 44, no. 12, pp. 2115–2133, 2001.

[78] S. Sanda, J. Bollyky, N. Standifer, G. Nepom, J. A. Hamerman, and C. Greenbaum, "Short-term IL-1β blockade reduces monocyte CD11b integrin expression in an IL-8 dependent fashion in patients with type 1 diabetes," *Clinical Immunology*, vol. 136, no. 2, pp. 170–173, 2010.

[79] T. Mandrup-Poulsen, "Interleukin-1 antagonists and other cytokine blockade strategies for type 1 diabetes," *The Review of Diabetic Studies*, vol. 9, no. 4, pp. 338–347, 2012.

[80] H. U. Kataoka and H. Noguchi, "ER stress and β-cell pathogenesis of type 1 and type 2 diabetes and islet transplantation," *Cell Medicine*, vol. 5, no. 2-3, pp. 53–57, 2013.

[81] B. Kutlu, A. K. Cardozo, M. I. Darville et al., "Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells," *Diabetes*, vol. 52, no. 11, pp. 2701–2719, 2003.

[82] A. K. Cardozo, H. Heimberg, Y. Heremans et al., "A comprehensive analysis of cytokine-induced and nuclear factor-κB-dependent genes in primary rat pancreatic β-cells," *Journal of Biological Chemistry*, vol. 276, no. 52, pp. 48879–48886, 2001.

[83] G. Kwon, J. A. Corbett, C. P. Rodi, P. Sullivan, and M. L. McDaniel, "Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of nuclear factor kappa B in the signaling mechanism," *Endocrinology*, vol. 136, no. 11, pp. 4790–4795, 1995.

[84] C. A. Dinarello, "Interleukin-1," *Cytokine & Growth Factor Reviews*, vol. 8, no. 4, pp. 253–265, 1997.

[85] K. Burns, F. Martinon, C. Esslinger et al., "MyD88, an adapter protein involved in interleukin-1 signaling," *Journal of Biological Chemistry*, vol. 273, no. 20, pp. 12203–12209, 1998.

[86] K. Burns, J. Clatworthy, L. Martin et al., "Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor," *Nature Cell Biology*, vol. 2, no. 6, pp. 346–351, 2000.

[87] T. T. Yamin and D. K. Miller, "The interleukin-1 receptor-associated kinase is degraded by proteasomes following its phosphorylation," *Journal of Biological Chemistry*, vol. 272, no. 34, pp. 21540–21547, 1997.

[88] S. A. G. Reddy, J. H. Huang, and W. S. L. Liao, "Phosphatidylinositol 3-kinase in interleukin 1 signaling. Physical interaction with the interleukin 1 receptor and requirement in NFκB and AP-1 activation," *Journal of Biological Chemistry*, vol. 272, no. 46, pp. 29167–29173, 1997.

[89] F. Ortis, P. Pirot, N. Naamane et al., "Induction of nuclear factor-κB and its downstream genes by TNF-α and IL-1β has a pro-apoptotic role in pancreatic beta cells," *Diabetologia*, vol. 51, no. 7, pp. 1213–1225, 2008.

[90] M. Flodströma, N. Welsh, and D. L. Eizirik, "Cytokines activate the nuclear factor κB (NF-κB) and induce nitric oxide production in human pancreatic islets," *FEBS Letters*, vol. 385, no. 1-2, pp. 4–6, 1996.

[91] B. Emanuelli, M. Glondu, C. Filloux, P. Peraldi, and E. van Obberghen, "The potential role of SOCS-3 in the interleukin-1β-induced desensitization of insulin signaling in pancreatic beta-cells," *Diabetes*, vol. 53, Supplement 3, pp. S97–S103, 2004.

[92] C. Bruun, P. E. Heding, S. G. Rønn et al., "Suppressor of cytokine signalling-3 inhibits tumor necrosis factor-alpha induced apoptosis and signalling in beta cells," *Molecular and Cellular Endocrinology*, vol. 311, no. 1-2, pp. 32–38, 2009.

[93] F. Ortis, A. K. Cardozo, D. Crispim, J. Störling, T. Mandrup-Poulsen, and D. L. Eizirik, "Cytokine-induced proapoptotic gene expression in insulin-producing cells is related to rapid, sustained, and nonoscillatory nuclear factor-κB activation," *Molecular Endocrinology*, vol. 20, no. 8, pp. 1867–1879, 2006.

[94] D. Kägi, A. Ho, B. Odermatt, A. Zakarian, P. S. Ohashi, and T. W. Mak, "TNF receptor 1-dependent β cell toxicity as an effector pathway in autoimmune diabetes," *The Journal of Immunology*, vol. 162, no. 8, pp. 4598–4605, 1999.

[95] H. Hsu, J. Huang, H. B. Shu, V. Baichwal, and D. V. Goeddel, "TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex," *Immunity*, vol. 4, no. 4, pp. 387–396, 1996.

[96] A. Devin, A. Cook, Y. Lin, Y. Rodriguez, M. Kelliher, and Z. G. Liu, "The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation," *Immunity*, vol. 12, no. 4, pp. 419–429, 2000.

[97] H. E. Kim, S. E. Choi, S. J. Lee et al., "Tumour necrosis factor-α-induced glucose-stimulated insulin secretion inhibition in INS-1 cells is ascribed to a reduction of the glucose-stimulated Ca^{2+} influx," *Journal of Endocrinology*, vol. 198, no. 3, pp. 549–560, 2008.

[98] M. A. Farrar and R. D. Schreiber, "The molecular cell biology of interferon-γ and its receptor," *Annual Review of Immunology*, vol. 11, no. 1, pp. 571–611, 1993.

[99] W. T. Windsor, L. J. Walter, R. Syto et al., "Purification and crystallization of a complex between human interferon γ receptor (extracellular domain) and human interferon γ," *Proteins: Structure, Function, and Genetics*, vol. 26, no. 1, pp. 108–114, 1996.

[100] G. Tau and P. Rothman, "Biologic functions of the IFN-γ receptors," *Allergy*, vol. 54, no. 12, pp. 1233–1251, 1999.

[101] J. Briscoe, D. Guschin, N. C. Rogers et al., "JAKs, STATs and signal transduction in response to the interferons and other cytokines," *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, vol. 351, no. 1336, pp. 167–171, 1996.

[102] K. Takeda and S. Akira, "STAT family of transcription factors in cytokine-mediated biological responses," *Cytokine & Growth Factor Reviews*, vol. 11, no. 3, pp. 199–207, 2000.

[103] H. S. Kim, M. S. Han, K. W. Chung et al., "Toll-like receptor 2 senses β-cell death and contributes to the initiation of autoimmune diabetes," *Immunity*, vol. 27, no. 2, pp. 321–333, 2007.

[104] H. I. Callewaert, C. A. Gysemans, L. Ladrière et al., "Deletion of STAT-1 pancreatic islets protects against streptozotocin-induced diabetes and early graft failure but not against late rejection," *Diabetes*, vol. 56, no. 8, pp. 2169–2173, 2007.

[105] F. Moore, N. Naamane, M. L. Colli et al., "STAT1 is a master regulator of pancreatic β-cell apoptosis and islet inflammation," *Journal of Biological Chemistry*, vol. 286, no. 2, pp. 929–941, 2011.

[106] C. A. Gysemans, L. Ladriere, H. Callewaert et al., "Disruption of the γ-interferon signaling pathway at the level of signal transducer and activator of transcription-1 prevents immune destruction of β-cells," *Diabetes*, vol. 54, no. 8, pp. 2396–2403, 2005.

[107] G. Pérez-Arana, M. Blandino-Rosano, A. Prada-Oliveira, M. Aguilar-Diosdado, and C. Segundo, "Decrease in β-cell proliferation precedes apoptosis during diabetes development in Bio-Breeding/Worcester rat: beneficial role of Exendin-4," *Endocrinology*, vol. 151, no. 6, pp. 2538–2546, 2010.

[108] D. L. Eizirik and M. I. Darville, "beta-cell apoptosis and defense mechanisms: lessons from type 1 diabetes," *Diabetes*, vol. 50, Supplement 1, pp. S64–S69, 2001.

[109] C. Holohan, E. Szegezdi, T. Ritter, T. O'Brien, and A. Samali, "Cytokine-induced β-cell apoptosis is NO-dependent, mitochondria-mediated and inhibited by BCL-X_L," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 2, pp. 591–606, 2008.

[110] W. S. Alexander, "Suppressors of cytokine signalling (SOCS) in the immune system," *Nature Reviews Immunology*, vol. 2, no. 6, pp. 410–416, 2002.

[111] C. Gysemans, H. Callewaert, L. Overbergh, and C. Mathieu, "Cytokine signalling in the β-cell: a dual role for IFNγ," *Biochemical Society Transactions*, vol. 36, no. 3, pp. 328–333, 2008.

[112] M. Flodstrom-Tullberg, D. Yadav, R. Hagerkvist et al., "Target cell expression of suppressor of cytokine signaling-1 prevents diabetes in the NOD mouse," *Diabetes*, vol. 52, no. 11, pp. 2696–2700, 2003.

[113] M. M. W. Chong, Y. Chen, R. Darwiche et al., "Suppressor of cytokine signaling-1 overexpression protects pancreatic β cells from $CD8^+$ T cell-mediated autoimmune destruction," *The Journal of Immunology*, vol. 172, no. 9, pp. 5714–5721, 2004.