Intraventricular T3 reverses chronic restraint stress-induced depressive-like behaviors: Inhibition of NF-κB/ NLRP3 inflammasome pathway in the hippocampus

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Abstract

In this study, the effects of triiodothyronine (T3) were evaluated on the NLR family pyrin domain containing 3 (NLRP3) inflammasome complex formation in the rat's hippocampus with restraint stress-induced depressive-like behaviors.Thirty-six Wistar male rats were randomly allocated to following groups: Control, Model, and Model + T3. In the Model or Model+T3 group, a single dose of PBS or T3 was administered into the lateral ventricle. Depressive-like behaviors were induced by chronic restraint stress. The forced swimming (FST), tail suspension (TST), and open field (OFT) tests were used to investigate the depression. The rats were sacrificed, and brain tissues were stored for molecular and pathological evaluations. Chronic stress increased the immobility of rats in the Model group according to FST, TST, and OFT ($P < 0.05$). T3 significantly improved depressive-like behaviors ($P < 0.05$). The gene expression and protein level of hippocampal nuclear factor kappa B (NF-κB), NLRP3, apoptosis-associated speck-like protein (ASC), and Caspase-1 significantly increased in the Model group compared to the control group ($P < 0.05$). The reduced hippocampal levels of NF-κB, NLRP3, ASC, and Caspase-1 were observed in the T3 group compared to the Model group ($P < 0.05$). The Nissl staining of the CA1 region showed an increased number of dark neurons ($P < 0.05$) and reduced pyramidal layer thickness ($P < 0.05$) in the Model group. These histopathological alterations were changed by T3 administration compared to the Model group ($P < 0.05$). The findings confirmed the therapeutic effects of intraventricularly T3 on depressive-like behaviors induced by restraint stress via surviving pyramidal neurons of the CA1 region and inhibition of NF-κB/NLRP3 inflammasome pathway.

Introduction

The function of several organs and systems, especially the central nervous system (CNS) and the endocrine system, can be affected by unpredictable and undesirable stressful conditions. Chronic and long-term stress contributes to the induction of a wide range of mental disorders, e.g., anxiety and depression [1]. Depression is an alteration in cognitive behavior and mood, featured by cognitive dysfunction, pleasure loss, negative mood, social isolation fatigue, sleep disorders, appetite loss, and other metabolic changes [2, 3]. Among different regions of the brain, it has been shown that reduced volume of the hippocampus is strongly correlated with the severity of depression [4]

Long-term unpredicted and repeated stress triggers a cascade of inflammatory responses in the brain [5]. A growing body of evidence from preclinical and clinical studies indicates that he depression is attributed to the higher levels of cytokines, such as interleukin (IL)-6, IL-1β in blood and cerebrospinal fluid [6]. Besides, the elevated levels of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6 in brain regions, including the hypothalamus, hippocampus, and/or prefrontal cortex, have been reported following depression [7, 4]. The nucleotide-binding domain, leucine-rich repeat (NLR) pyrin domain protein 3 (NLRP3) inflammasomes, an intracellular multi-protein complex, is activated in various neurological and psychological disorders [8]. Full assembly and activation of the NLRP3 complex depend on two important factors of nuclear factor kappa B (NF-κB) signaling pathways and interaction of NLRP3 protein with the rest of the inflammasome machinery, including apoptosis-associated speck-like protein

containing a caspase recruitment domain (ASC) and procaspase 1 [9]. After activation of complex, procaspase 1 is cleaved into caspase 1, which activates IL-1β from the pro-IL-1β [10]. A growing body of evidence confirmed that NF-κB is an important mediator of stress-induced neurological impairments [11].

Several animal models have been established to investigate the pathological events related to the progression of depression or effects of antidepressants. The exposure of animals to stressors leads to alterations that can be seen in pateints with depression [12]. Chronic restraint stress is a low-cost stressor that induces a series of behavioral changes similar to those seen in humans [13].

Different classes of antidepressants have been developed based on the hypothesis that depression, as a neuroendocrine disorder, result from an imbalance in the monoamine neurotransmitters [14]. However, several side effects, e.g., sexual dysfunction, cardiovascular disease, and osteoporosis, have been reported for these antidepressants [15, 16]. Recently, the relationship between thyroid axis abnormalities and psychiatric disorders has been proven [17]. Triiodothyronine (T3) and thyroxine (T4), two major thyroid hormones (THs), are secreted from the thyroid gland [18]. Both hyper and hypothyroidism patients experience numerous neuropsychiatric manifestations, such as anxiety and depressive disorders [19]. Accordingly, low T3 syndrome, described as decreased levels of peripheral T4 to T3 in the normal production of TH, has been recorded in patients with psychiatric depression [20]. The treatment of patients with THs to manage their mood disorders, particularly depressive disorders, was reported in numerous studies [21]. It w as also demonstrated that T3 could augment and accelerate treatment response with antidepressants [22]. T3 and T4 can cross the blood-brain barrier (BBB) via monocarboxylate 8 (MCT8), a specific transporter of THs [23]. T3 binding to TH receptor (TR) isoforms, predominantly TRα1 and TRβ1, mediates its function. Notably, TRα1 is located on nearly all types of neurons [24]. Thus, the adequate local concentration of T3 is important for TRs activation [25]. T3 (the active and less TH) contributes to numerous biologic functions in normal and pathologic conditions via different mechanisms. THs regulate the expression of oxidative stress products, glutamate, and neurotrophic factors in neurological disorders [26]. Furthermore, the anti-inflammatory effect of THs was reported in several studies [27, 28].

Due to the beneficial effects of T3, we used a single dose of intraventricular (IVC) T3 to evaluate its anti-depressive effects in a chronic restraint stress model. To understand the mechanisms of effects of T3 as a therapeutic approach, we designed an experiment to examine: (1) depressive-like behaviors of animals, (2) activation of the NLRP3 inflammasome, and (3) pathological alterations via Nissl staining.

Method And Material

Animals

The present research was approved by the Ethics Committee of Animal Experiments at Sadat City University, Sadat City, Egypt and performed based on the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). It was funded by Taif University (Supporting Project

number: TURSP-2020/99). In the present study, 36 male Wistar rats (weighing 150-220 g) were kept at the standard temperature (23+1°C), 12/12 h light/ dark cycle with ad libitum access to food and water.

Experimental design

The study protocol was designed as demonstrated in Figure 1. Rats were randomly allocated to three groups (n = 12 in each group): Control (healthy animals), Model: with rats subjected to restraint stress and IVC injected with phosphate-buffered saline (PBS) [29], Model+T3: rats subjected to restraint stress and injected IP with T3. The rats in the Model and Model+T3 groups were exposed to stress. For this purpose, each rat was placed in a plastic cylinder (3 h/day from 8:00 am to 11:00 am) for 14 consecutive days [29]. Twenty-four hours after model induction, the animals in the Model+T3 group were injected with IVC T3 (a single dose of 25 ug/kg body). T3 was dissolved in dimethyl sulfoxide and diluted with PBS (a total volume of 5 ul) to be injected into the right ventricle via a Hamilton syringe (bregma: ML = -1.8 mm, AP = -0.9 mm, and DV = 3.5 mm deep from the dura) [26]. The equal volume of PBS was injected into the right ventricle of rats in the Model group. The rats were examined via forced swimming test (FST), tail suspension test (TST), and open field test (OFT). In the end, brains of rats were used for various molecular (n=8 in each group) and histological (n=4 in each group) studies. Fresh samples of the hippocampus were extracted immediately after sacrificing, put in a freezing tube, and kept at -80°C. Also, for histological studies, the total brain was isolated after prefixation. The prefixation procedure was done via the transcranial perfusion of normal saline to remove the brain's blood, followed by 4% paraformaldehyde perfusion (PFA, Sigma) via the same route. The post-fixed was done by using a 10% formalin at 4° C for 72 h.

Behavioral study

Forced swimming test (FST)

A glass cylinder with a height of 80 cm and a diameter of 30 cm filled with 40 cm of water (25°C) was used to evaluate the rats' behavior for two consecutive swimming sessions of training and test. The cylinder was filled with tap water (23 ± 1 °C), and water depth was adjusted according to the animal size. Therefore, rats could not touch the bottom of the cylinder. At first, for training, each rat was placed in the cylinder for 10 minutes and forced to swim. After 24 h, the procedure was repeated for 5 min period as a test session. In this session, the animal behavior was video-recorded by a blind observer, and the immobility, latency, swimming, and climbing times were recorded [30]. An enhanced time of immobility time was considered as depressive-like behavior.

Tail Suspension Test (TST)

One day after FST, TST was performed. Briefly, each rat was suspended via its tails using adhesive tape to a horizontal bar for 6 min (2 min for the adaptation and 4 min for the main test). The test was performed by a blind observer, and the immobility time was recorded.

Open Field Test (OFT)

The OFT was performed to evaluate the depressive-like behavior, as reported previously [31]. Briefly, the open-field apparatus (80 cm×80 cm×50 cm^2) is consists of a floor divided into 25 equal squares. Each rat was placed in the center of the open field individually and allowed to explore for 5 min freely, and the behavior was recorded. The time of immobility was evaluated for a 5 min period. After finishing the test, the rat was placed in the home cage by the experimenter. After each test, the apparatus was cleaned with 90% ethanol to remove olfactory cues.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

The fresh samples were used for quantitative real-time PCR (qRT-PCR) assay (n= 4 in each group) of NF-κB, NLRP3, Caspase-1, and ASC. In summary, total RNA was isolated from samples by using the Tripure Isolation Reagent (Roche Applied Science, Peuzberg, Germany) according to the instruction. NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific) was used for evaluation of its purity. For complementary DNA synthesis, PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Shiga, Japan) was used for reverse transcription. The PCR primers used are shown in Table 1. The StepOnePlus Real-Time PCR machine was carried out to run the reactions in triplicates. beta-actin (β-actin) was used to normalize the gene expression, and the relative fold change expression of genes was calculated via the 2-ΔΔCt method [32].

Western Blot

Using western blot technique, the isolated hippocampal samples were used (n = 4 in each group) to determine the synthesis of inflammasome proteins. The samples were lysed via a lysis buffer (RIPA) and centrifuged. Total Protein Kit, Micro (Sigma, USA) was used to detect the total protein concentration. After protein denaturation, 5 µg of protein were loaded on 10% SDS-PAGE, and separated proteins were put on polyvinylidene difluoride transfer membranes (Sigma, USA) and then incubated for one hour with specific primary antibodies (Novus Biologicals, USA). Then, the procedure was followed by incubation of membranes for one hour with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Germany). Protein bands were detected by the luminescent substrate solution (Sigma, USA). Finally, to quantify the specific bands, ImageJ software (NIH, USA) was used. GAPDH (Thermoscientific, USA) was used for normalization [33].

Nissl Staining (Cresyl Violet Staining)

After fixation and tissue processing (n = 4 samples in each group), 5-um-thick coronal sections cut by microtome (Leica Biosystems, Milan, Italy) and mounted on the slides. After subjecting to Nissl staining, four random sections were picked for light microscopy observation. Photomicrographs of the CA1 region were prepared under an optical microscope (Labomed, USA). The number of dark neurons (intensely stained neurons) were calculated in each photomicrograph.

Statistical analysis

Data were analyzed via SPSS software (V22.0) and expressed as mean ± SD. The analyses were carried out using the one-way analysis of variance (ANOVA) and followed by post hoc Tukey's comparison. *P* value <0.05 was considered significan.

Results

Behavioral tests

To determine the effects of T3 on depressive-like behaviors, we performed behavioral investigations, including FST, TST, and OFT. Our results showed that exposure of animals to restraint stress (Model and Model+T3 groups) enhanced the immobility and latency time (*P<0.05,* fig. 2, a-b) and reduced the swimming and climbing time (*P<0.05,* fig. 2, c-d) in FST evaluations and treatment with T3 could improve the FST behaviors compared to the Model group (*P<0.05,* fig. 2, a-d). Increased immobility time in TST and OFT was also observed in the Model and Model+T3 groups compared to the Control group (*P<0.05,* fig. 2, e-f). Treatment of rats with T3 decreased the mean time of immobility in both tests compared to the Model group (*P<0.05,* fig. 2, e-f).

qRT-PCR analysis

The mean gene expression of hippocampal NF-κB, NLRP3, ASC, and Caspase-1 was evaluated in each group. A significant increase in the gene expression of hippocampal NF-κB, NLRP3, ASC, and Caspase-1 was observed in the Model and Model+T3 groups compared to the Control group (*P< 0.05,* fig. 3, a-d). The gene expression of hippocampal NF-κB, NLRP3, Caspase-1, and ASC was significantly reduced in the Model+T3 group compared to the Model group (*P < 0.05,* fig. 3, a-d).

Western Blot Analysis

The mean protein levels of NF-κB, NLRP3, Caspase-1, and ASC were investigated in the hippocampal region. Fig. 4 shows a significant increase in the protein levels of hippocampal NF-κB, NLRP3, ASC, and Caspase-1 in the Model and Model+T3 groups compared to the Control group (*P< 0.05,* fig. 4, a-e). The protein levels of hippocampal NLRP3, Caspase-1, ASC, and significantly reduced in the Model+T3 group compared with the Model group (*P < 0.05,* fig. 4, a-e).

Histopathological Examination

For the evaluation of pathological changes, Nissl staining was performed. The pathological alterations of CA1 were investigated in terms of the number of dark neurons and the pyramidal cell layer thickness. A significantly increased number of dark neurons in the CA1 region was reported in the Model and Model+T3 groups compared to the Control group (*P < 0.05,* fig. 5, a-d). The number of dark neurons reduced in the Model+T3 group compared to the Model group (*P < 0.05,* fig. 5, a, b, c, & d). The pyramidal cell layer thickness significantly decreased in the CA1 region in the Model and Model+T3 groups

compared to the Control group (*P < 0.05,* fig. 5, a, b, c, & e). Furthermore, there was a significant increase in the pyramidal cell layer thickness in the Model+T3 group compared to the Model group (*P < 0.05,* fig. 5, a, b, c, & e).

Discussion

The results of the current study revealed that treatment with a single dose of T3 ameliorated depression-like behaviors induced by the restraint stress. The IVC microinjection of T3 could alleviate the activity of inflammasome through reducing the gene expression and protein concentrations of NF-κB, NLRP3, ASC, and Caspase-1 in the hippocampus. Additionally, T3 improved the histopathological changes of the CA1 hippocampal region.

In the present study, we exposed animals to chronic immobility stress to induce depressive-like behavior. According to the findings, chronic stress increased the immobility behavior of animals. The enhanced levels of NF-κB, NLRP3, ACs, and Caspase-1 were observed in the hippocampus. Pathologically, the number of dark neurons and the pyramidal layer thickness decreased in the Model group. These findings indicated that neuroinflammation after animals to the stressor induced the histopathological and behavioral alterations in animals. Rodent models of chronic restraint stress with more than 35 years of use for modeling psychological disease have been employed to investigate biological and behavioral manifestations of clinical depressive disorders in humans [34, 35]. Depression is a psychological disorder associated with several pathological events, such as neural death in the hippocampus [36]. The neural loss of the hippocampus is correlated with decreasing the hippocampal volume in depressive disorders [37]. According to the literature, psychological or physical stressors may be followed by the activation of inflammation and subsequent production of inflammatory cytokines, resulting in functional and structural changes in neurons [38]. Several studies have demonstrated that the interaction between the CNS and the immune system plays a critical role in stress-induced neuroinflammation and depression [39]. Therefore, the peripheral administration of immunostimulants is often used for the induction of inflammation-related depression in animal models [40]. The anxious and depressive behaviors have been shown to be associated with the enhanced levels of TNF-α in the hippocampus and striatum [41].

Among a wide range of cytokines, IL-1β seems to play an important role in the pathological features of depressive-like behavior caused by stress [42]. It has been demonstrated that IL-1β reduced neurogenesis in human hippocampal progenitor cells through stimulation of the kynurenine signaling pathway, a common finding in depression. Both inhibitors of this pathway and traditional antidepressants can modulate this effect [43]. The NLRP3 inflammasome is a major intercellular mediator of IL-1β maturation and secretion [44]. The activation of the NF-*κ*B/NLRP3 inflammasome signaling pathway has been proven in animal models of depression and patients with major depressive disorders [45, 46]. Furthermore, NF-*κ*B signaling and NLRP3 inflammasome have previously been suggested as a link between the immune system and stressors, the potential pathologic features in developing depressive disorder [14, 46]. Activation of NF-κB induces the transcription of pro-IL-18, pro-IL-1β, and NLRP3 [47]. Besides, some factors, e.g., overload calcium, reactive oxygen species, and ATP, can directly stimulate the

NLRP3 inflammasome [48, 49]. On the other hand, the inflammation is commonly related to oxidative stress. It seems that oxidative damages such as DNA oxidation and lipid peroxidation contribute to pathological features of neuropsychiatric diseases [50]. Additionally, the association between glutamate and inflammation has been well demonstrated [35]. A large number of clinical studies suggest that the pathophysiology of depression is associated with dysfunction of the predominant glutamatergic system, known as the glutamate hypothesis [51]. The NLRP3 inflammasome also is responsible for pyroptosis and apoptosis in hippocampus neurons mediates depressive-like behavior in diabetic mice [52]. Therefore, the increased number of dark neurons is associated with the activation of the NF-κB/ NLRP3 inflammasome pathway.

In the current study, we used a single dose of IVC T3 following the induction of depressive-like behaviors. The administration of T3 could inhibit the activation of the inflammasome complex in the hippocampal region via reducing the NF-κB, NLRP3, ASC, and Caspase-1 concentration and prevent the pathological features seen in the hippocampal region. According to FST, TST, and OFT findings, these alterations were correlated with improved depressive-like behavior. Therfore, T3 could survive the neurons of the CA1 region through anti-inflammatory properties. According to the evidence from preclinical and clinical THs, abnormalities are seen in depressive disorders [53, 54]. Therefore, the regulation of the thyroid axis may help to improve the symptoms of depression. T3 alone or in combination with antidepressants has been used to treat patients with depressive disorders [54]. THs are essential for brain development in both fetal and postnatal periods [24]. Previously, it was recorded that the levels of T3 and T3/T4 ratio are higher in the brain of mammalian compared to the systemic circulation [55]. T3 has been shown to augment or accelerate the treatment of depressive disorders [56]. This hormone acts as a stimulator of gene expression and regulates the cellular energy for metabolism and neurogenesis in the CNS [57]. Recently, the IVC injection of T3 was revealed to survive neurons of the CA1 hippocampal region in the rat model of brain stroke via the upregulation of neurotrophic factors [58]. In another study, exogenous T3 exerted neuroprotective features via modulation of the NF-κB pathway (anti-inflammatory effects) and enhanced levels of neurotrophic factors in a traumatic brain injury [59]. Moreover, the administration of T3 could diminish alcoholic liver disease and ischemia-reperfusion-induced liver via modulation of the NLRP3 signaling pathway [60, 61]. In accordance with our results, T3 may suppress the NF-κB/ NLRP3 inflammasome pathway through anti-inflammatory features, contribute to reverses the neural death in the hippocampus, and improve the depressive like behavior in rats exposed to chronic stress.

Conclusion

In summary, restraint stress induction activated the NLRP3 inflammasome in the hippocampus region, correlated with pathological and behavioral alterations. To attenuate these changes, we used a single dose of T3 via the IVC route. The findings of our study indicated that T3 regulated the gene expression and protein levels of NF-κB, NLRP3, ASC, and Caspase-1 in the hippocampus and accordingly improved the pathological and behavioral alterations induced by restraint stress in the rat model. T3 could survive the pyramidal neurons of the hippocampus through the regulation of NF-κB/ NLRP3 inflammasome pathway. These results confirmed the beneficial effects of T3 in the treatment of depressive symptoms.

Declarations

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Conflicts of interest/Competing interests:

The authors declare that they have no competing interests.

Availability of data and material:

Data are however available from the authors upon reasonable request

Authors' contributions:

LH and AME contributed substantially to the conception and design of the study. AME, and TM contributed to perform the experiment, LH contributed to analyze the data, TM and AME drafted or provided critical revision of the article. LH provided the final approval of the version to publish. All authors discussed the results and contributed to the final manuscript.

Ethics approval:

This research was approved by the Ethics Committee of Animal Experiments at Sadat City University, Sadat City, Egypt and performed based on the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Consent for publication:

N/A

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Tables

Table 1. List of primers

Gene	Forward primer	Reverse Primer
NF-κB	AATTGCCCCCGGGCAAT	TCCCGTAAACCGGCGTAA
NLRP3	GGAGTGGATAGGTTTGCTGG	GGTGTAGGGTCTGTTGAGGT
Caspase-1	GTGGAGAGAAAGAAGGAGTGGT	GATGAGTGACTGAATGAAGAGG
ASC	TCTGGAGGGGTATGGCTTGG	GAGTGCTTGCCTGTGTTGGT
b-actin	ACAACCTTCTTGCAGCTCCTC	CTGACCCATACCCACCATCAC

Figures

Figure 1

Study protocol.

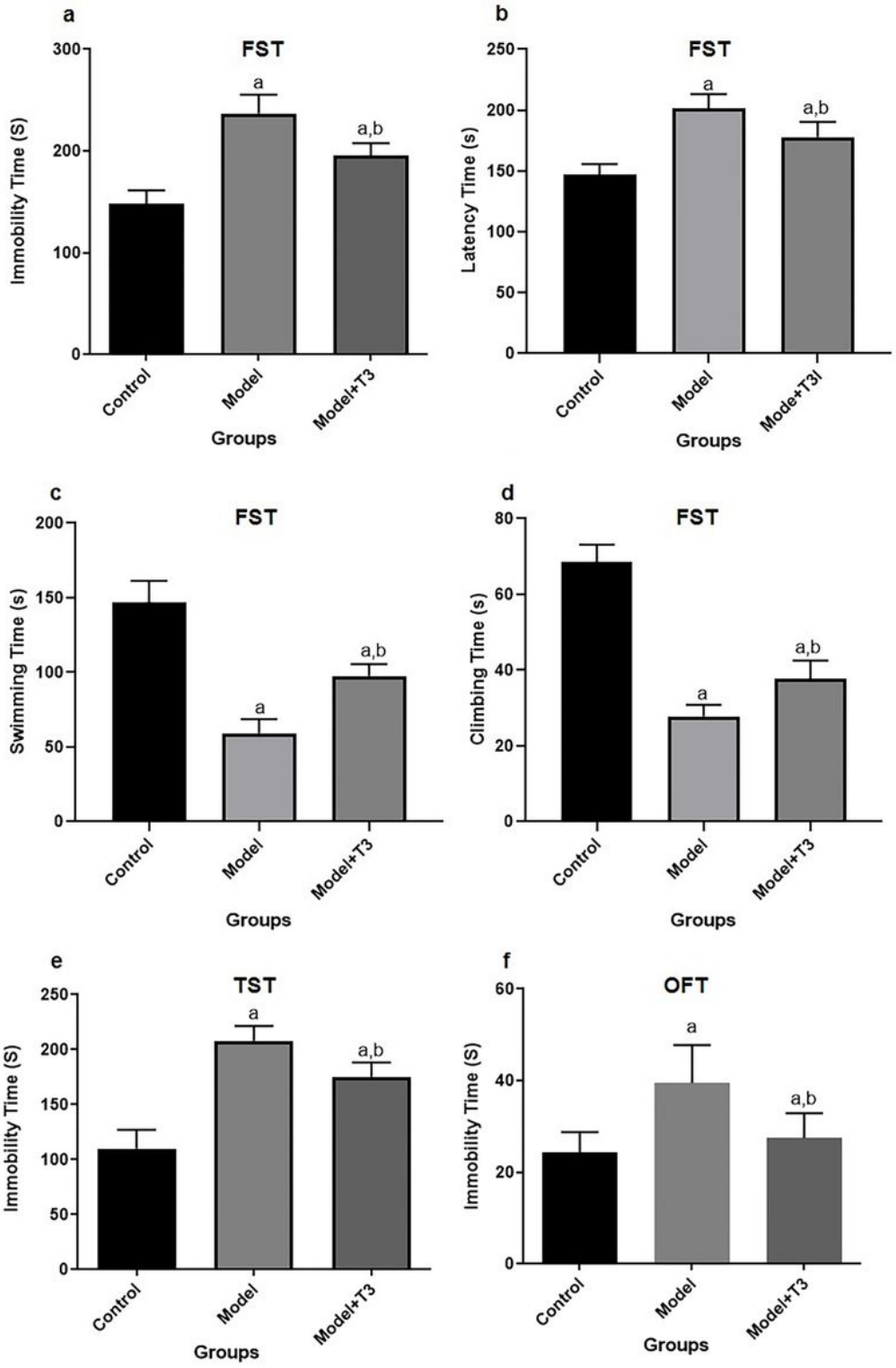

Figure 2

Effects of T3 on depressive-like behavior following restraint stress in rats. a. Immobility time (s), b. Latency time (s), c. Swimming time (s), and d. Climbing time (s) in FST, e. Immobility time (s) in TST, and f. Immobility time (s) in OFT in. a $P< 0.05$ compared to the Control group, b $P< 0.05$ compared to the Model group. Control: Normal and healthy rats, Model: Restraint stress group received PBS, Model+T3: Restraint stress group received T3.

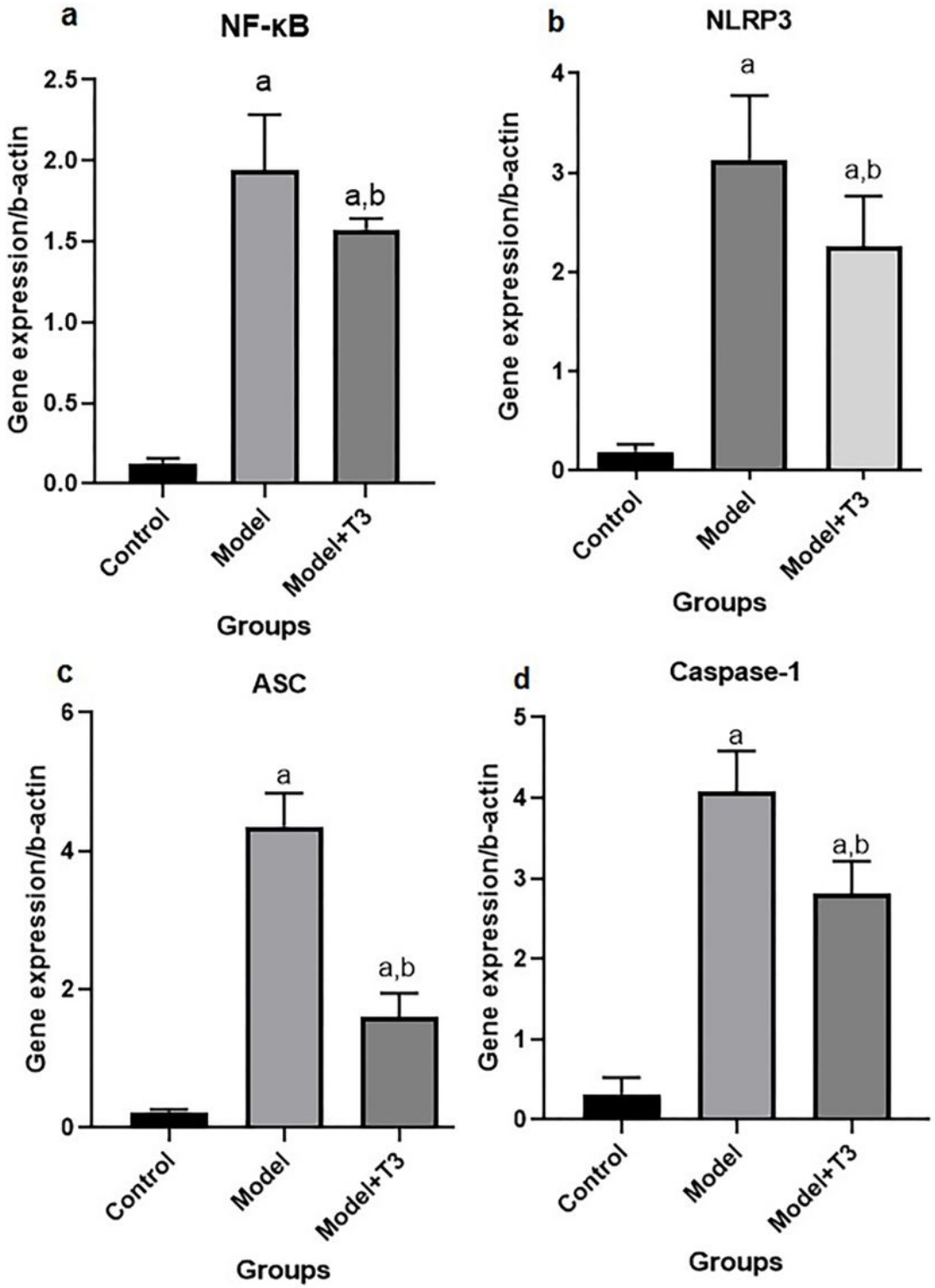

Figure 3

Effects of T3 on gene expression of a. NF-κB, b. NLRP3, c. Caspase-1, and d. ASC in the hippocampus of rats with depressive-like behavior. a $P< 0.05$ compared to the Control group, b $P< 0.05$ compared to the Model group. Control: Normal and healthy rats, Model: Restraint stress group received PBS, Model+T3: Restraint stress group received T3.

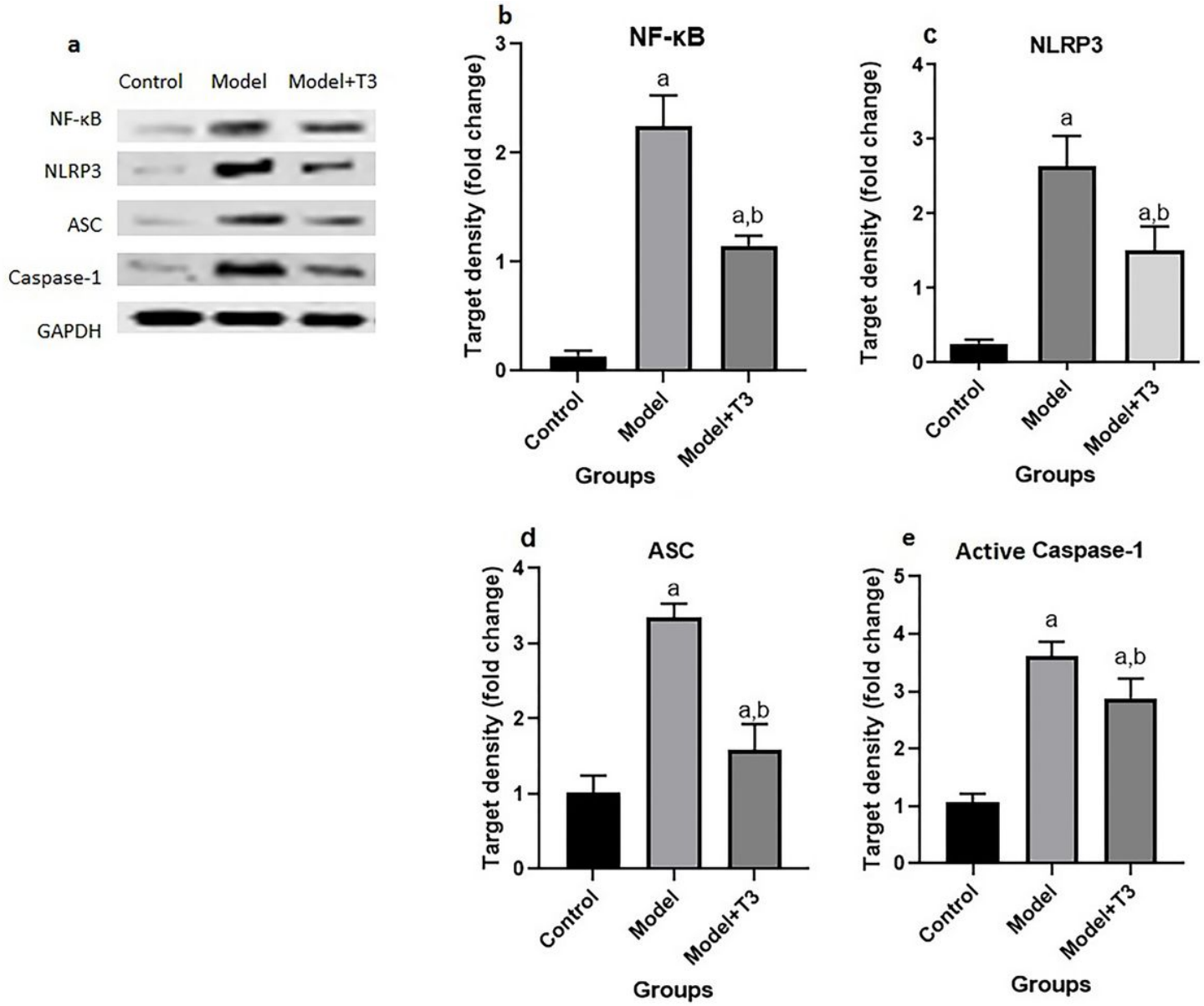

Figure 4

Effects of T3 on protein levels of a, b. NF-κB, a, c. NLRP3, a, d. Caspase-1, and a, e. ASC in the hippocampus of rats with depressive-like behavior. a $P< 0.05$ compared to the Control group, b $P< 0.05$ compared to the Model group. Control: Normal and healthy rats, Model: Restraint stress group received PBS, Model+T3: Restraint stress group received T3.

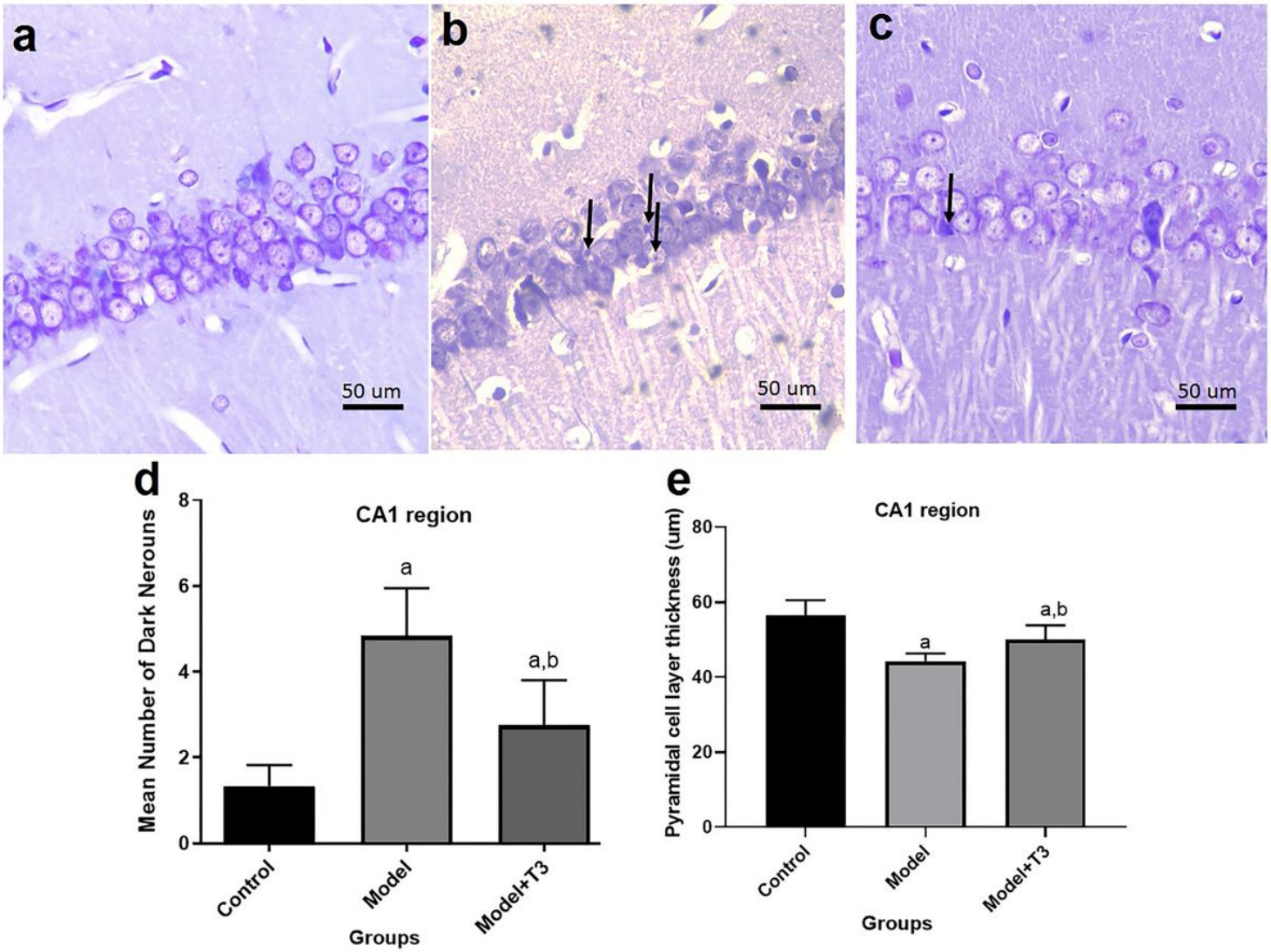

Figure 5

Effects of T3 on histopathological changes of the CA1 region of the hippocampus of mice with depressive-like behavior. a, b, c. Nissl staining (scale bar: 50 μm), d. Comparing the mean number of dark neurons (arrows) of the CA1 region in different groups. a $P< 0.05$ compared to the Control group, b $P< 0.05$ compared to the Model group. Control: Normal and healthy rats, Model: Restraint stress group received PBS, Model+T3: Restraint stress group received T3.