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Baicalein mitigates cognitive impairment in chronic stress rat model by downregulating TLR4/NF-kB signaling pathway

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- Baicalein
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- Stress

Abstract

Introduction: Chronic stress can significantly impair memory and learning processes by changing the hippocampus and other brain regions. Baicalein (Baic) has been shown to possess anti-inflammatory and antioxidant qualities. **Objective**: to demonstrate the neuroprotective mechanism of Baic in stress-induced cognitive impairments. **Material & methods**: Thirty male Wister albino rats were split into three: control, stress, and stress + bait. Rats underwent NOR and EPM tests, hippocampus gene expression of TLR4, NF-kB, and BDNF was evaluated, and serum corticosterone, hippocampal MDA, SOD, TNF-α, and IL-6 were examined.TLR4 and NF-kB immunoreaction for hippocampus were performed. **Results :** In contrast to the control group, there were higher hippocampal TLR4 and NF-kB immunoreaction and a significantly lower discrimination index of the NOR test, time in the open arms of EPM, SOD, and gene expression of BDNF of stress group demonstrated cognitive impairment and significantly increasing serum corticosterone, hippocampal MDA, TNF-α, IL-6, and hippocampal gene expression of TLR4 and NF-kB. Baic significantly improved the alterations in the brain caused by stress. **Conclusion:** Baic reduced stress-induced cognitive deficits by down-regulation of the TLR4/NF-kB signaling pathway as well as neurotrophic, antioxidant, and anti-inflammatory mechanisms.

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Introduction

A state of compromised homeostasis brought on by internal or external stressful events is known as stress. It triggers the sympathetic nervous system and HPA-axis, which leads to an adaptive physiological and behavioral reaction [1].

According to reports, stress has an impact on all facets of life. Additionally, it is well established that stress can negatively impact health both directly and indirectly by altering neuroendocrine and autonomic processes. One important factor connected to stress-induced behavioral issues is the activation of the HPA axis, which raises corticosterone levels in the brain [2].

The levels of stress hormones in the blood can rise as a result of ongoing stress. The hippocampus, a limbic area crucial to memory and learning, is one of the most susceptible to stress. Stress produces long-term potentiation and cognitive deficits by activating hippocampus glucocorticoid receptors, increasing neuronal metabolism, decreasing cell survival and neurogenesis, and causing dendritic atrophy. It is well suited to stress, which can affect hippocampal-dependent cognitive processes, especially memory and learning [3].

Chronic stress can significantly contribute to memory and learning disruption by changing brain regions, particularly the hippocampus. Numerous animal models have been created to investigate how long-term stress affects behavioral and cognitive processes. Even though cognitive dysfunction brought on by long-term stress is very common, finding appropriate preventative strategies or treatments is still very difficult [4].

Long-term changes in several neurosystems linked to anxiety, depression, cognition, and sleeplessness are brought on by chronic stress, which also impairs brain function. Long-term stress exposure lead to the development of neurological conditions, such as Alzheimer's disease (AD), and the progression of dementia [5].

The pathophysiology of neurodegenerative illnesses, including cognitive problems, has been linked to increased ROS generation and decreased antioxidant levels. Chronic stress exposure can change the ratio of antioxidants to oxidants, causing a significant production of free radicals and reducing the effectiveness of antioxidants. Rats exposed to prolonged stress have oxidative damage in their hippocampal regions as a result of increased lipid peroxidation and decreased antioxidant levels[5].

Changes in hippocampus synaptic plasticity can cause memory impairment when oxidative stress levels are high. Furthermore, there is proof that antioxidants can lessen the harm that oxidative stress causes to neurons. Consequently, using antioxidant drugs could be a good way to stop and treat cognitive impairment [5].

Toll-like receptor 4 (TLR4) has a significant impact on inflammation and are key modulators of the innate immune system.TLR4 is one of the TLR family members that is extensively expressed in CNS.As the CNS's initial line of defense against the immune system, microglia are crucial to neuroinflammation. Microglia have high levels of TLR4 expression, which activates microglia. Neurologic impairment results from an inflammatory cascade response triggered by activated microglia[6].

TLR4 agonists cause the NF-κB to enter the nucleus, which increases the transcription of proteins linked to inflammation and causes hippocampus neurons to produce inflammatory

factors [7].The stimulation of ROS triggers the TLR4/NF-κB pathway and modulates many inflammatory genes alike[8]..

Widely expressed in the hippocampus, BDNF is a neurotrophic growth factor that plays a crucial function in controlling neuronal transmission and plasticity while also enhancing memory consolidation [9]..

It has been demonstrated that BDNF has both neurogenic and neuroprotective properties. Depletion of neurotrophic factors causes AD-related disorders, such as tau hyperphosphorylation, Aβ buildup, and synaptic dysfunction, according to recent studies conducted in animal models [10]..

The traditional Chinese herb baicalein, (Baic), is made from the root of Scutellariabaicalensis Georgi. It is a member of the flavonoid class and has several biological properties, such as anti-inflammatory, antioxidant, and anti-apoptotic properties. Baic significantly enhances memory and learning and may be used as a medication to prevent and treat central nervous system diseases. Baic helps people with cognitive impairment [7]..

Additionally, Baic modulates the TLR4/MyD88/NF-κB signaling pathway to decrease glial cell activation and proinflammatory factor release [11]..

It has been demonstrated that Baic can pass across the blood-brain barrier and may have pharmacological effects by acting directly in brain nuclei. Furthermore, it has been documented that Baic reduces hippocampus neurogenesis via increasing BDNF signaling and regulating oxidative stress [12]..

This study was created to demonstrate the neuroprotective impact of Baic on cognitive deficits brought on by stress and the underlying processes at play.

Materials and methods

Animals. The study was authorized by Menoufia University's Institutional Animal Care and Use Committee (IACUC), with IRB No.: MUFS/F/GE/5/25.The experiment involved thirty male Wister albino rats weighing between 200 and 250 grams. The experimental methods were conducted in accordance with the ARRIVE standards. The rats were housed in 80 x 40 x 30 cm wire-mesh cages.

Experimental groups. (10/group):

1. Control group: rats were not exposed to stress, For 4 consecutive weeks, rats received daily intragastric 0.5% sodium carboxymethylcellulose (CMC-Na) (cat. no. 21904; MilliporeSigma)
2. Stress group: for four weeks rats were exposed to chronic stress as proven in stress protocol mentioned below, furthermore rats received daily intragastric 0.5% CMC-Na for 4 consecutive weeks.
3. Stress + Baic group: rats were given 100 mg/kg/d Baic intragastrially for four weeks [7,13],starting on the first day of the experiment, and they were also exposed to a chronic stress paradigm for four weeks. Baic (Must, Chengdu, China) was dissolved in 0.5% CMC-Na and given one hour prior to stress exposure

Chronic unpredictable mild stress (CUMS) induction

Subjects in this animal model are subjected to a variety of mild and erratic stressors on a daily basis. These stress-inducing treatments were administered in the following order: 5 minutes of swimming in cold water (4 °C), 1 minute of tail pinching (1 cm from the end of the tail), 24 hours

of continuous lighting, 24 hours of wet cages, 24 hours of tilting cage (45°), 5 minutes of heat stress (45 °C), 20 minutes of shaking (one shake per second), 24 hours of food deprivation, 2 hours of confinement in a restrainer, and 24 hours of water deprivation. With the exception of food and water deprivation, which were performed twice, all of the stressors were repeated three times in the same order over the course of four weeks [14,4].

After the four weeks, a neurobehavioral examination was performed on each rat. The rats were then fasted for the entire night. The serum was then separated for the determination of serum corticosterone after retroorbital blood samples were obtained at 10 a.m. After being anesthetized, the rats' cervical vertebrae were dislocated and lengthened to scarify them. The brain was extracted and washed with phosphate buffer saline (pH 7.4).The left hemisphere was weighed and then divided in half, with one half being used for biochemical analysis and the other half for RT-PCR study. The right hemisphere of the hippocampal tissues was fixed in formalin for histological and immunohistochemical analysis.

Neurobehavioral tests

Novel Object Recognition: item in a regulated environment. Each rat underwent a three-day testing process that included training, testing, and habituation. During the habituation phase, rats were placed in an open-field apparatus that measured 50 cm by 50 cm by 40 cm. They were allowed to adjust to their environment for 10 minutes. For the first five minutes of training, we put two identical items in each rat's room. Each rat was kept in the chamber for five minutes following the item exchange, which occurred twenty-four hours later, during the testing phase. We put the stopwatch to work.[= (familial object exploration time–novel object exploration time)/total exploration time×100%] is one way to calculate the discriminating index.[15]

Elevated Plus Maze (EPM) Test: In this experiment, we used an apparatus to have the rats identify a plus sign in order to assess their anxiety-like behavior. Each rat was placed in the middle of the apparatus and allowed ten minutes to explore the labyrinth. The animals' movements were monitored by an overhead security camera. The duration of time spent in the open arms mazes was meticulously documented. The duration and the intensity of anxiety-like behavior were inversely correlated [16].

Hippocampal homogenate preparation

Each hippocampal specimen was homogenized. The tissue was centrifuged for 17 minutes at 13,000 rpm in centrifuge and stored for biochemical analysis.

Biochemical analysis

The ELISA kit (catalog No. CSB-E07014r, CUSABIO Life Science Inc., Washington, DC, USA) was used to measure the serum corticosterone level. However, rat ELISA kits (TNF-α: ERT2010 1, Assaypro LLC, Saint Charles, Missouri, USA, IL-6: ab100772, Abcam, Cambridge, UK) were utilized to assess the quantities of IL-6 and TNF-α in the hippocampus. Hippocampal MDA and SOD were measured using colorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt) in accordance with the manufacturer's instructions.

Quantitative assay of hippocampal TLR4, NF-κB and BDNF genes expression using RT-PCR.

The QiagenRN easy plus Universal Kit from the USA was used to prepare hippocampal samples for total RNA isolation. The quality and purity of the RNA were then assured. Until it was required, RNA was stored at -80 °C. The first step then was to create cDNA in a single cycle on an Applied Biosystems 2720 heat cycler in Singapore using the QuantiTect Reverse Transcription Kit, which is produced by Qiagen in the USA. In RT-PCR processes, GAPDH primers were used as an RNA loading control. cDNA amplification was the second step. SensiFASTTMSYBR Lo-ROX Kit, USA, used cDNAin SYBR green-based quantitative real-time PCR for Relative Quantification (RQ) of TLR4, NF κB, and BDNF gene expression using the following primers (Midland, Texas):

The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC)., the reverse primer was (GAGCAGTCATGTCCTTGGGT). The forward primer for TLR4 was (TCAGCTTTGGTCAGTTGGCT), and the reverse was (GTCCTTGACCCACTGCAAGA)

The forward primer for BDNF was GCTGCCTTGATGTTTACTTTG and reverse ATGGGATTACACTTGGTCTCGT.

Finally, the 2.0.1 version of the Applied Biosystems 7500 software was used to finish the data analysis. The RQ of TLR4, NF κB, and BDNF gene expression was conducted using a comparative ΔΔCt technique, which normalizes the amount of target gene (TLR4, BDNF, and NF κB) mRNA to an endogenous reference gene (GAPDH) and compares it to a control.[13]

Histological study:

After being cleansed and dehydrated, the hippocampal samples were embedded in paraffin blocks. Five to seven μm thick serial coronal slices were cut and dyed using: For routine histological investigation, use the Hx. & E.stain.

Immunohistochemical studies:

Blocks of paraffin were deparaffinized and then rehydrated using decreasing alcohol grades.TLR4 [(Catalog No. GB11186; Servicebio, Wuhan, Hubei, China)], NF-kB (monoclonal, dilution 1:200, Abcam), were added after the endogenous peroxidase activity was blocked with 3% H2O2 in methanol. The incubation period was overnight.

Statistical analysis

SPSS version 23 was used to process the data (SPSS Inc., USA).To make sure all of the data sets were normally distributed, the Shapiro-Wilk test was used. The mean ± SD was used to express the data. A post-hoc Tukey test and a one-way ANOVA were used to determine the significance of group differences. For statistical significance, P values below 0.05 were used.

Results

While the Stress group's discrimination index of the NOR test, time in the open arms of EPM, hippocampal SOD, and hippocampal gene expression of BDNF values were substantially lower than control, the Stress serum corticosteron, hippocampal MDA, hippocampal TNF-α, hippocampal IL-6, and hippocampal gene expression of TLR4 and NF κB were substantially higher than the control .While the discrimination index of the NOR test, time in the open arms of EPM, hippocampus SOD, and hippocampus gene expression BDNF values of the Stres + Baic group were significantly higher than those of Stress, they were still significantly lower hippocampal MDA,

hippocampal TNF-α, hippocampus IL-6, serum corticosterone and hippocampus gene expression of TLR4 and NF κB. (Table 1)

Table (1): The measured discrimination index of NOR test, time in the open arms of EPM, serum corticosteron, hippocampal MDA, SOD, TNF-α, IL-6, hippocampal TLR4 gene expression, hippocampal NF-κB gene expression and hippocampal BDNF gene expression in all studied groups

	Control group	Stress group	Stres +Baic group
Discrimination index of NOR Test	61.2±3.78	17.1±3.6 *	41.9±6.2*#
Time in the open arms of EPM	120.8±6.33	43.1± 2.8*	81.8± 6.35*#
serum corticosteron (ng/Ml)	51±4.58	130± 3.27*	86± 5.33*#
Hippocampal MDA (nmol/ gm. Tissue)	3.99 ±0.12	16.33± 1.33*	7.9± 0.72*#
Hippocampal SOD (U/gm. Tissue)	4.55±0.1	1.08±0.08*	2.78±0.03*#
Hippocampal TNF-α (ng/ml)	22.99±2.33	64.89±4.88*	41.25±3.9*#
Hippocampal IL-6 (pg/mL)	140.98±6.18	251.8±9.13*	173.9±2.12*#
Hippocampal TLR4 gene expression	1	3.98±0.06*	2.01±0.09*#
Hippocampal NF-κB gene expression	1	2.99±0.08*	1.62±0.04*#
Hippocampal BDNF gene expression	1	0.41±0.07*	0.71±0.05*#

* Significant compared with control, # Significant compared with Stress

Histological results

The three layers that comprised the control group's hippocampus were the inner polymorphic, middle pyramidal, and outer molecular layers, all of which showed a typical histological appearance. The hippocampal tissue integrity was significantly reduced in the Stress group. With hyperchromatic shrunken nuclei and mildly clogged blood vessels, the pyramidal cells seemed decrepit. The pyramidal layer looked to have improved with Stress+Baic because many pyramidal cells had big vesicular nuclei, prominent nucleoli, and basophilic cytoplasm, but some of them were remained degraded. (X400). (Fig. 1)

Immunohistochemical results:

The Stress group's immunostain % in NF-kB-stained sections was significantly higher ($p<0.05$) than that of the control (83.5±0.03 vs. 10±4.33).Though it was remained higher than the control, the stress+ Baic exhibited a considerable decrease in this percentage to the Stress group (29.2±.02 vs. 83.5±0.03). (Fig. 2 A-D)

The immunostain percentage in TLR4-stained sections was considerablly higher ($p<0.05$) in the Stress group than in the control group (85.4±0.24 vs. 11.8±0.16).Though it was remained higher than the control group, the stress+ Baic exhibited a considerable decrease in this proportion to the Stress (26.2±0.23 vs. 85.4±0.24).(Fig. 2 E-H)

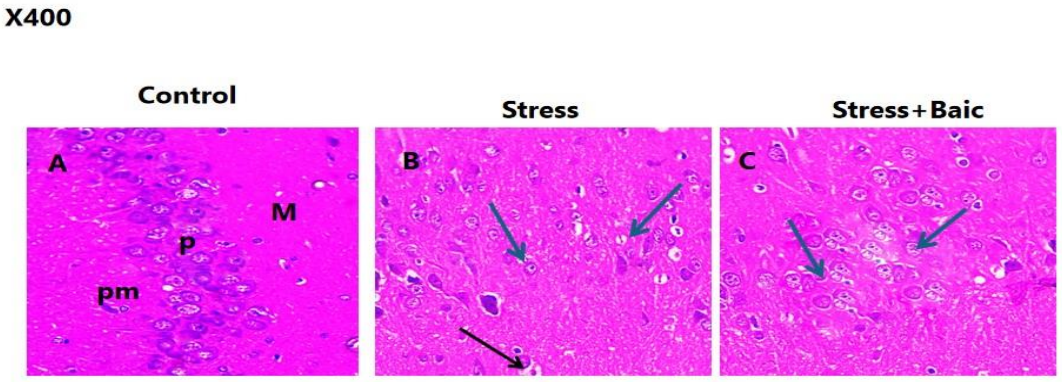

Fig. 1: Photomicrographs of the hippocampus of the different groups showing: (A) The hippocampus of the control group was formed of of the three layers; outer molecular (M), middle pyramidal (P) and inner polymorphic (Pm) layers that appeared with normal histoloical picture. (B) In the Stress group, the hippocampus showed marked loss of the hippocampal tissue integrity. The pyramidal cells appeared degenerated with hyperchromatic shrunken nuclei (blue arrows) and mild congested blood vessele was appeared (black arrow). The Stress+Baic showed an apparent improvement of the pyramidal layer as many of the pyramidal cells appeared normal with basophilic cytoplasm, large vesicular nuclei and prominent nucleoli but still some of them still degenerated (blue arrows). (X400).

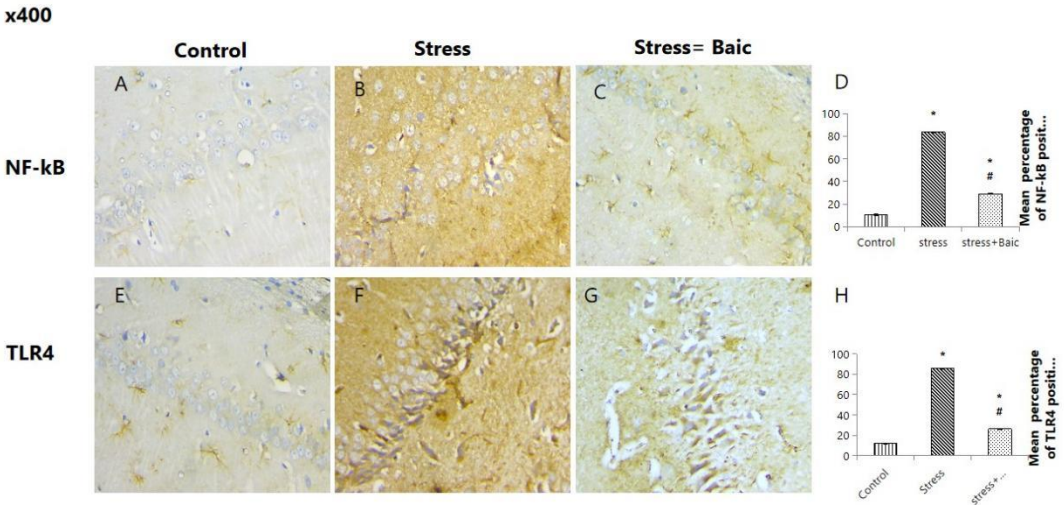

Fig. 2: Representative micrographs of the different experimental groups showing a considerable upregulation of the NF-kB (A-D) and TLR4 (E-H) immunoreaction in the Stress group but a significant downregulation in the Stress+ Baic group.(X400).

Discussion

The findings implied that the cognitive damage brought on by long-term stress was lessened by the administration of Baic. According to earlier research, long-term stress has a significant regulatory role in the emergence of cognitive impairment. The hippocampal region, the primary brain region linked to mood and thought processes, is particularly vulnerable to long-term stress. Cognitive impairment results from oxidative damage in the hippocampus caused by stress-induced ROS generation. Chronic stress also encourages changes in the hippocampus's structure and function, which impairs cognitive function [5]. One of the best methods is the CUMS model, which simulates chronic stress in real life by exposing animals to a range of unpredictable, mild stressors over an extended period of time. The long-term impacts of chronic stress on mood, cognition, and biochemistry are well captured by this model [4].

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The goal of the current study was to investigate Baic's potential protective effects against stress-induced memory impairments in rats. This study's key discovery was that stress-induced memory impairments, which were linked to a decrease in hippocampal biochemical markers, were reversed after 28 days of Baic treatment. As demonstrated by a substantial drop in recognition index (RI) in NOR and time spent in the open arms of the EPM test compared to control, our research validated the concept that stress affects anxiety and memory function, which is consistent with other research[3].

Baic improved the cognitive impairments brought on by ongoing stress, which is consistent with earlier research [7].Baicalein's anti-oxidant, anti-inflammatory, and acetylcholinesterase-inhibitory qualities all contribute to its neuro-protective effects [17].

According to Song et al., Baic significantly enhanced stress-induced histological changes in the hippocampal region, demonstrating its neuro-protective qualities [7].

As demonstrated by findings that the higher levels of corticosterone in the stress group disrupted the circadian regulation of corticosterone secretion, chronic stress can cause or worsen a disruption in the activity of the HPA axis. Affective behaviors and psychosomatic diseases are intimately linked to elevated GC levels, which alter brain function and make it more difficult to regulate physiological and behavioral reactions to stimuli. Additionally long-term stress alters HPA axis function, resulting in morphological changes in the prefrontal cortex, hippocampus, and hypothalamus as well as in a number of neurotransmitters, weight loss, and behavioral abnormalities [12].

Our results, which are consistent with other studies of enhanced stress-induced corticosterone production, demonstrate that chronic stress considerably raises serum corticosterone levels. This increase is a normal physiological reaction to extended stress, triggered by the HPA axis and leading to increased production of corticosterone to deal with stresses [4].But compared to the stress group, Baic's corticosterone levels dropped, which is consistent with other research[12].

Stress-induced memory impairments have a complex process. Notably, the findings showed that the hippocampi of stress-exposed rats had considerably more MDA and lower SOD than control rats, indicating a triggered oxidative stress response that was consistent with earlier research [18].

Confirmed by our histological findings, the stress group demonstrated that oxidative stress-induced lipid peroxidation causes changes in synaptic membrane depolarization, malfunctions in neurotransmission systems, and may result in the atrophy or even death of hippocampus neurons. Furthermore, it has been demonstrated that maintaining adequate antioxidant levels is essential for the development of spatial memory since their shortage results in hippocampus apoptosis and the failure of synaptic plasticity mechanisms, both of which are linked to impairments in spatial memory [19].

When combined, these findings pointed to a new function for antioxidants in regulating these memory impairments. As previously stated, our research demonstrated that Baic cotreatment reduced the oxidative stress response triggered by stress, which in turn reduced the ensuing

pathogenic events that underlie stress-induced memory impairment [20].

The compound's ability to scavenge free radicals and have an antioxidant effect, as well as its encouragement of transcription and enzymatic activity of numerous antioxidant enzymes, including SOD, may be the cause of Baic's antioxidant capacity. Furthermore, research has demonstrated that Baic can improve antioxidant properties by increasing the expression of antioxidant enzyme genes. Additionally, Baic's antioxidant properties prevent mitochondrial dysfunction by increasing Nrf2 [20].

Numerous inflammatory cytokines have been shown to be released as a result of prolonged stress. According to certain research, TLR4 has a role in stress-induced processes. Being a transmembrane receptor, TLR4 can be triggered by "danger" signals like stress and damage in addition to inflammatory ones. In contrast to the control, TNF-α and IL-6 levels progressively rose and peaked four weeks after stress, which is consistent with other research [21].

Additionally, the stress group's TLR4/NF-kBhippocampus gene expression and immunoreaction were significantly higher than control. The TLR4/NF-κB pathway was implicated in the pathophysiology of chronic stress, according to Wang et al. [21].

Our findings indicated that when compared to the stress group, rats treated with Baic had significantly lower hippocampal TLR4/NF-kB gene expression and immunoreaction, as well as lower levels of the inflammatory cytokines IL-6 and TNF-α. This suggests that Baic may target TLR4/NF-kB to reduce stress-induced cognitive deficit, which is consistent with other research [22].In a prior study, baic therapy reduced glial cell activation and pro-inflammatory molecules by blocking the TLR4/MyD88/NF-κB signaling pathway [23].

Widely expressed in the hippocampus, BDNF is a neurotrophic growth factor that plays a crucial function in controlling neuronal transmission and plasticity while also enhancing memory consolidation [3].

According to earlier research, the results presented here demonstrated a considerably lower BDNF in the hippocampus of stress-exposed rats as compared to control rats, indicating their involvement in the pathophysiology of chronic stress-induced cognitive deficits [3].

Furthermore, BDNF can change several signaling pathways related to learning and memory. The BDNF signaling pathways have been linked to tau protein phosphorylation, neuroinflammation, Aβ plaque formation, and neuronal death [24].

It's interesting to note that stress cotreatment with Baic dramatically offset the noticeable BDNF level alterations, demonstrating their role in its neuroprotective effect in this rat model and corroborated other research [13].Baic'santioxidant ability may be the reason for its attenuating effect on BDNF [13].It has previously been established that oxidative stress and the decline in BDNF levels are related [3].

Conclusion

Baic reduced stress-induced cognitive deficits by down-regulation of the TLR4/NF-kB signaling pathway as well as neurotrophic, antioxidant, and anti-inflammatory mechanisms.

Conflict of interest

The authors declares that there is no conflict of interest

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