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Apelin attenuates depressive-like behavior and neuroinflammation in rats co-treated with chronic stress and lipopolysaccharide

Zi-xuan Zhang[a,b,1], E. Li[c,1], Jian-ping Yan[e], Wan Fu[e], Pei Shen[e], Shao-Wen Tian[d,*], Yong You[a,*]

[a] Department of Neurology, The Second Affiliated Hospital, Hainan Medical University, Haikou, Hainan 571199, PR China
[b] Department of Neurology, XiangTan Central Hospital, Xiangtan, Hunan 411100, PR China
[c] Institute of Neuroscience, Chinese Academy of Sciences, CAS Center for Excellence in Brain Science and Intelligence Technology, Shanghai 200031, PR China
[d] Department of Physiology, Hengyang Medical College, University of South China, Hengyang, Hunan 421001, PR China
[e] Department of Neurology, The First Affiliated Hospital, University of South China, Hengyang, Hunan 421001, PR China

A B S T R A C T

Several experimental studies have proved that activation of neuroinflammation pathways may contribute to the development of depression, a neuropsychiatric disorder disease. Our previous studies have shown the antidepressant properties of apelin, but the mechanism was unkown. This study was performed to verify whether the antidepressant effect of apelin was related to its anti-inflammation effect in the central nervous system. To achieve our aim, we selected the co-treatment of chronic stress and LPS to induced an inflammatory process in rats. The effect of this co-treatment was evaluated through the expression of inflammatory markers and glial cell activation. LPS injection co-treated with unpredictable chronic mild stress resulted in the activation of microglial cell and astrocyte, expression of inflammatory markers and depressive behaviors. Treatment with apelin significantly attenuates the deleterious effects in these rats. Our results showed that apelin improved depressive phenotype and decreased the activation of glial cells in stress co-treatment group. The down-regulations of p-NF-κB and p-IKKβ suggested that the effects are possibly mediated by inhibition of the NF-κB-mediated inflammatory response. These findings speculated that intracerebroventricular injection of apelin could be a therapeutic approach for the treatment of depression, and the antidepressant function of apelin may closely associated with its alleviation in neuroinflammation.

1. Introduction

Depression is a severe chronic disorder with high morbidity and mortality. The main symptoms of depression include feelings of sadness, loneliness and guilt, lack of enthusiasm, sleep disorders, and self-contempt (Miller et al., 2014; Ceretta et al., 2012a, 2012b, 2012c). Depression leads to poor quality of life and great social and financial burdens (Miret et al., 2013). Although antidepressant drugs and electroconvulsive therapy may play an important role in the clinic, their effects are still limited (Nestler et al., 1989). For example, almost two-thirds of patients with depression are insensitive to that therapy (Nestler et al., 1989). Therefore, we must determine the pathogenesis of depression and seek more effective antidepressant drugs as well as therapies with less side effects and better therapeutic effects than the current treatment options.

Preclinical and clinical evidence suggests that neuroinflammation is critically implicated in the pathological process of depression (Raison and Miller, 2015; Young et al., 2014; Haapakoski et al., 2015; Strawbridge et al., 2015). Animal research has shown that microglia and astrocytes, the immune cells in the central nervous system (CNS), are potential mediators of inflammatory alterations in depression (Romina et al., 2013). Activation of microglia and astrocytes in response to inflammatory stimuli is able to increase the release of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (Diz-Chaves et al., 2012; Zhao et al., 2014a). NF-κB has been known to act as essential transcription factors for the expression of inflammatory mediators, such as iNOS, COX-2, IL-1β and TNF-α (Lawrence and Fong, 2010), which lead to depressive-like behavior (Biesmans et al., 2013; Yuan et al., 2015). In addition, NF-κB signaling may play a critical role in activation of glia cells (Kim et al., 2014), indicating that the activation of NF-κB signaling precedes microglial and astroglial activation. These cytokines impact the neurotransmitters in the hippocampus and neuronal apoptosis in the brain and trigger the activation of microglia, astrocytes and inflammatory processes, all of which are potential mechanisms of depression (Smith, 1991; Dowlati et al., 2010; Kim et al., 2007). In clinical research, patients with inflammatory disorders often exhibit depressive symptoms (Ceretta et al., 2012a, 2012b, 2012c). This relationship is bidirectional, as patients with depressive disorder display increased serum levels of IL-1β, IL-6, IL-8, and TNF-α (Howren et al., 2009). One of the antidepressant effects of fluoxetine is

* Corresponding authors.
E-mail addresses: 47089887@qq.com (S.-W. Tian), youy_usc@qq.com (Y. You).
[1] Authors contributed to the paper equally.

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associated with a decrease in the expression of inflammatory cytokines in the CNS (Orio et al., 2010), and cyclooxygenase inhibitors have been shown to decrease the expression of inflammatory cytokines in the periphery to ameliorate depressive symptoms in depressed patients (Na et al., 2014; Köhler et al., 2014). Therefore, anti-inflammatory drugs may become the new antidepressant drugs.

Stress is an important cause of mental illnesses such as depression. Almost all psychiatric patients have experienced stressful events (Kessler et al., 2010). Stress is known to contribute to the development of depression. Antidepressant drugs such as selective serotonin reuptake inhibitors and serotonin and norepinephrine reuptake inhibitors reduce microglial activation and inhibit inflammatory responses (Ohgi et al., 2013; Tynan et al., 2012). In animal research, stress has been shown to lead to a series of depressive behaviors indicated by weight loss, adrenal weight gain, an increase in immobility time in the forced swimming test and a change in relative sucrose intake in the sucrose preference test (Gibson-Smith et al., 2015; Forbes et al., 1996; Clark et al., 2016; Sakhaee et al., 2017; O'Keane et al., 2012). In addition, stress sensitizes hippocampal neurons to a proinflammatory stimulus (Espinosa-Oliva et al., 2011a, 2011b; de Pablos et al., 2006), accompanied by microglia and astrocyte activation and inflammatory cytokine expression (Shih et al., 2015; Lawrence and Fong, 2010).

Apelin is a newly identified type of adipocyte factor. Apelin was first isolated from the stomach extract of cattle, and its precursor protein has 77 amino acid residues (Tatemoto et al., 1998). This preproapelin can be proteolyzed by angiotensin-converting enzyme 2 and cleaved into four active peptides including apelin-12, apelin-13, apelin-17 and apelin-36. Apelin-13 is one of the most biologically active peptides (Hosoya et al., 2000). Apelin peptide and its receptor APJ are widely distributed in the cardiovascular system, lung tissue, lymphocytes, kidney and pancreas, as well as the hypothalamus, hippocampus, limbic structures, midbrain, caudate nucleus and other brain regions in the central nervous system, in which it plays important physiological functions (O'Carroll et al., 2013; O'Carroll et al., 2003; Reaux et al., 2001). Apelin also plays an important role in physiological responses to homeostatic perturbations, including cardiovascular disease control, water balance, HPA axis regulation and metabolic homeostasis (O'Carroll et al., 2013). In vitro and in vivo, apelin has been reported to exert an anti-neuroinflammatory effect in myocardial infarction (Xin et al., 2015), suppress anxiety (Telegdy and Jászberényi, 2014), modulate learning (Telegdy et al., 2013; Han et al., 2014) and memory and exert a neuroprotective function (Yang et al., 2015).

Our previous studies demonstrated that apelin-13 exerts an antidepressant effect, as shown by the reversal of despair behavior in the forced swim (FS) test and escape failures in the learned helplessness (LH) test (Li et al., 2016). We compared the antidepressant effect of apelin-13 and imipramine, a positive control, certified the idea that apelin-13 has an antidepressant effect. Apelin-13 also has a protective effect against corticosterone-induced apoptosis in PC12 cells (Zou et al., 2016). However, the effect of apelin on unpredictable chronic mild stress (UCMS) and lipopolysaccharide (LPS) co-treatment in depression and whether the effect is associated with inflammation inhibition are still unclear. Our previous studies mainly focused on acute stress, while this study examined chronic stress. Studies have found that patients with inflammatory diseases who have undergone stressful events have a high risk of depression. Therefore we examined the effect of apelin on rats exposed to chronic stress and lipopolysaccharide co-treatment to determine whether apelin can exert an antidepressant effect on this model and the possible mechanism.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–220 g) were purchased from the Hunan SJA Laboratory Animal Co., Ltd., Changsha, Hunan, China. The rats were housed individually (temperature of 22 ± 2 °C and 60% relative humidity) where food and water were freely obtained. Rats were maintained on a 12-h light/dark cycle, with lights on at 7 A.M. The rats were individually handled (5–6 min per day) for 1 week to habituate them to the experimenter. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the University Animal Care and Use Committee.

2.2. Surgical procedures and cannulas placement verification

The operation was the same as we described previously (Li et al., 2016). Specifically, rats were given an intraperitoneal injection of sodium pentobarbital (45 mg/kg) and then placed in a stereotaxic apparatus (Stoelting Co. USA). A cannula (Mode 62,001, RWD Life Science Co., Ltd., Shenzhen, China) was aseptically implanted into the right lateral ventricle (1.0 mm posterior, 2.0 mm lateral and 4.0 mm ventral to the bregma) (Paxinos and Watson, 1998). The cannula was fixed to the skull with three stainless steel screws and dental cement. After the operation, rats were placed in cages alone. The rats were allowed to recover for 5 to 7 days after operation. We use the angiotensin II drinking test to verify successful placement of the cannula (Li et al., 2016; Choi et al., 2003). The rats who drank > 5 ml of water in 20 min in response to intracerebroventricular (i.c.v.) injection of angiotensin II were considered to have correct cannula placement in this study.

2.3. Drugs and intracerebroventricular (i.c.v.) injection

Apelin-13 and lipopolysaccharide (LPS, from *E. coli*, serotype 026:B6) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Apelin-13 and LPS were freshly dissolved in sterile saline before each experiment. The doses of each i.c.v. injection of apelin-13 and LPS were both 2 μg. The i.c.v. injection procedure was similar to what we previously described (Li et al., 2016). Specifically, the rat was held gently throughout the injection, which lasted at least 4 min. After drug injection, the needle was kept in the cannula to ensure that the drugs were completely injected into the cella lateralis. The effective doses of apelin-13 and LPS were selected on the basis of previous studies (Telegdy et al., 2013; de Pablos et al., 2014).

2.4. Chronic stress and LPS co-treatment

LPS (characteristic components of the cell wall of Gram-negative bacteria) has been shown to induce inflammatory responses. Recent studies suggest that unpredictable chronic mild stress (UCMS) can strengthen LPS-induced inflammatory responses (Espinosa-Oliva et al., 2011a, 2011b; Couch et al., 2016). In this study, we chose the co-treatment of chronic stress and LPS as a stress. Rats were randomly divided into a stress group and non-stress group after 5 days of recovery from lateral ventricle cannula placement. UCMS was adapted from different models of variable stress (Gamaro et al., 2003; Konarska et al., 1990; Murua and Molina, 1992; Muscat et al., 1992; Papp et al., 1991; Willner et al., 1987) with modifications as reported previously (Espinosa-Oliva et al., 2011a, 2011b; de Pablos et al., 2006). Each stressor and duration of application each day are found in Table 1. Application of stress began at different times every day (between 08:00 and 20:00) to minimize predictability. Body weight was measured at the beginning and the end of the 10-day treatment period. The rats were decapitated 24 h after the last stress, and the hippocampus was removed from the brain. The hippocampus was stored at −80 °C and prepared for western blot.

2.5. Drug administration

We divided the stress group and the non-stress group into two subgroups at random according to the different treatments. Four groups

Table 1
Schedule of stressors.

Day	Stressor	Duration	Start Time
1	Forced swimming	10 min	9 a.m
2	Flash light	6 h	4 p.m.
3	White noise	4 h	11 a.m.
4	Tail clamping	1 min	9 a.m.
5	Flash light	6 h	5 p.m.
6	Forced swimming (4 °C)	10 min	10 a.m.
7	Wet bedding (during night cycle)	12 h	10 p.m.
8	Forced swimming	10 min	9 a.m.
9	Cage tilt (45°, during night cycle)	12 h	9 p.m.

of animals were established: V, non-stressed/vehicle group, which received a 9-day i.c.v. injection of 2 μl vehicle; A, non-stressed/apelin-13 group, which received a 9-day i.c.v. injection of 2 μl apelin-13; SLV, stressed/LPS/vehicle group, which received a single i.c.v. injection of 2 μl LPS (carried out after the application of the first stress), were stressed for 9 days and were given a daily dose of 2 μl vehicle for 9 days half an hour after stress exposure; SLA, stressed/LPS/apelin-13 group, which received a single i.c.v. injection of 2 μl LPS (carried out after the application of the first stress), were stressed for 9 days and were given a daily dose of 2 μl apelin-13 for 9 days half an hour after stress exposure (Fig. 1).

2.6. Behavioral methods

2.6.1. Forced swimming test (FST)

The FST is used to evaluate depressive-like states (Cryan et al., 2002). In our study, the FST was used as a stressor to induce depressive-like behavior and to evaluate the depressive-like state by analyzing the immobility time. Details of the instrument were described in a previous study (Tian et al., 2010). Specifically, the experimental device was an opaque plastic bucket with a diameter of approximately 40 cm and a height of approximately 80 cm. The depth of the water was approximately 18–22 cm to ensure that the rats were not able to stand directly in the water. The water temperature was kept at approximately 25 °C for 10 min (Bagci et al., 2016). Water was changed between subjects. The whole FST process was recorded by a camera positioned over the bucket, and the total immobility time (with the rat suspended and maintaining the head on the surface of the water while the extremities and the tail are relatively inactive) of each rat in the first 5 min was manually counted by two experimenters individually. Depressive-like behavior (behavioral despair) was defined as an increase in immobility time. This test was conducted on day 1 and day 8 of the stress procedures. We record the data as an assessment of the state of depression.

2.6.2. Sucrose preference test (SPT)

The SPT is a convenient and effective way to judge the depressive-like state. Depressive rats show a decline in sucrose water intake. Specifically, after rats adapted to the environment and the experimenters, the test was carried out in their cages. During the acclimatization period (3–5 days), rats were provided with 1.5% (*w/v*) sucrose and drinking water to adapt the rats to the sucrose water (Asor et al., 2013). In this stage, the two bottles were randomly placed at both ends of the cage, and their position was randomly changed every day to ensure a reliable basal sucrose preference measure. Then the test was carried out individually for each rat at one week before surgery, one day before surgery, one week after surgery (one day before the stress procedure) and on day 4 and day 9 of the stress procedure. At 8:00 p.m., the drinking water bottles were removed from the rat cages, and the rats were not allowed to drink water for 12 h. The next morning at 8:00 a.m., we randomly placed one bottle of 1.5% (*w/v*) sucrose and one bottle of drinking water on either end of the cage. The rats were exposed to the two water bottles for 4 h, and then both water bottles were removed at 12:00. The intake of the sucrose water and the tap water over the 4 h was recorded, and sucrose preference was calculated (Avital et al., 2011).

Sucrose preference = [sucrose water intake (g)]/[sucrose water intake (g) + drinking water intake (g)] × 100.

2.7. Western blot analysis

The western blotting procedure was the same as we described previously (Tang et al., 2005). The hippocampus was lysed in RIPA lysis buffer (APPLYGEN, Beijing, China). The homogenate was centrifuged at 12,000 ×*g* for 15 min at 4 °C, and the supernatant was collected. The protein concentration was determined using a BCA protein assay kit. Then, protein extracts were separated by electrophoresis on 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and then with a primary antibody overnight at 4 °C. The membranes were incubated with primary antibodies (diluted according to the instructions for the different proteins) at 4 °C for 1 h and then incubated with secondary antibodies for 2 h at room temperature. The protein bands were quantified by Quantity One imaging software (Bio-Rad, Hercules, CA, USA) and normalized against β-actin. Then all membranes were washed in TBST and incubated with HRP goat anti-rabbit IgG at a dilution of 1:3000 for 1 h at room temperature in TBST. The protein bands were visualized using western blotting chemiluminescence luminal reagent (Thermo pierce, Waltham, USA). This procedure was repeated in triplicate. The protein bands were quantified by Quantity One imaging

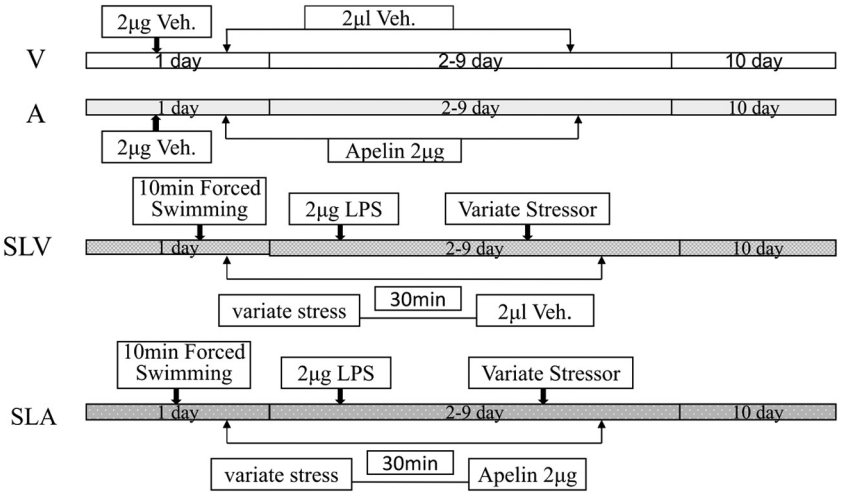

Fig. 1. Experimental groups and treatments. Intracerebroventricular injections of vehicle (Veh) or lipopolysaccharide (LPS) were given at day 1. In the stressed groups (SLV,SLA), intranigral injections were carried out after the application of the first stressor (10 min of forced swimming). In the SLA group, apelin-13 was injected every day 0.5 h before the stressor. V, non stressed / vehicle group; A, non stressed / apelin-13 group; SLV, stressed / LPS / vehicle group; SLA, stressed / LPS / apelin-13 group.

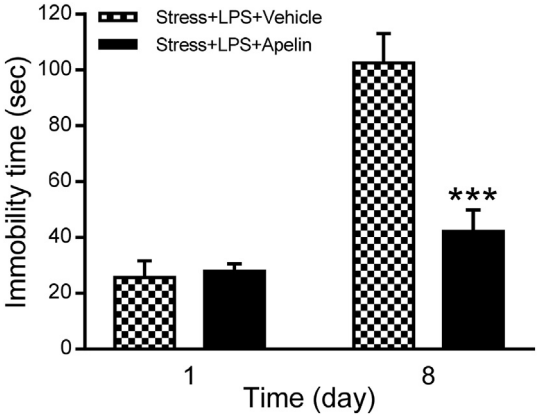

Fig. 2. Effect of Apelin-13 treatment on the immobility times of rats in the FST. Immobility times of rats in the FST following stress exposure for 1 day and 8 days. ***$p < .001$ compared with Stress + LPS + Vehicle rats. $N = 7$ per group, all values given are the mean ± SEM.

software (Bio-Rad, Hercules, CA, USA) and normalized against β-actin.

2.8. Statistical analysis

All experimenters were blinded with respect to treatment. Data were expressed as the mean ± standard error mean (SEM). Statistical analysis was analyzed by SigmaStat (system) and performed using unpaired one-way analysis of variance (ANOVA) or two-way RM-ANOVA with repeated-measures (treatment × time). p values of < 0.05 were considered to be statistically significant.

3. Results

3.1. Apelin-13 reverses the depressive phenotype in rats co-treated with chronic stress and LPS

In the forced swimming test (FST), animals show "despair" behavior as exhibited by immobility. To evaluate whether apelin-13 possesses an antidepressant-like activity, we measured the immobility time during the first 5 min in the FST (Fig. 2). A two-way RM-ANOVA of immobility time in the FST (Fig. 2) found no significant time effect ($F(2, 83) = 2.900$, $p = .065$), a significant treatment effect ($F(1, 6) = 14.310$, $P = .009$), time effect ($F(1, 27)) = 66.356$, $P < .001$) and interaction of treatment and time ($F(1, 27) = 34.970$, $P = .001$). Post hoc comparisons showed that treatment with apelin-13 significantly reduced the immobility time on day 8 ($p < .001$). The results suggest that apelin-13 improves depression behavior in rats co-treated with chronic stress and LPS.

Then we investigated the effects of apelin-13 on the sucrose preference test. The relative sucrose intake was detected in rats before stress exposure (0 days) and 4 and 9 days following stress exposure, with intake at 0 days used as a baseline. A two-way RM-ANOVA of relative sucrose intake (Fig. 3) found no significant effect of time ($F(2, 83) = 2.900$, $p = .065$) but a significant effect of treatment ($F(3, 24) = 6.557$, $p = 0.002$) and interaction between treatment and time ($F(6, 83) = 0.5.648$, $p < .001$). On day 4, post hoc comparisons showed that chronic stress and LPS co-treatment did not produce a significant decrease in sucrose intake ($p = .160$), but apelin-13 resulted in a significant increase ($p < .001$). On day 9, post hoc comparisons showed that chronic stress and LPS co-treatment produced a significant decrease in sucrose intake compared with control ($p = .004$), and this decrease was reversed by apelin-13 treatment ($p < .001$). The results suggest that chronic stress and LPS co-treatment caused depressive behavior, but apelin-13 improved the depression behavior in rats co-treated with chronic stress and LPS.

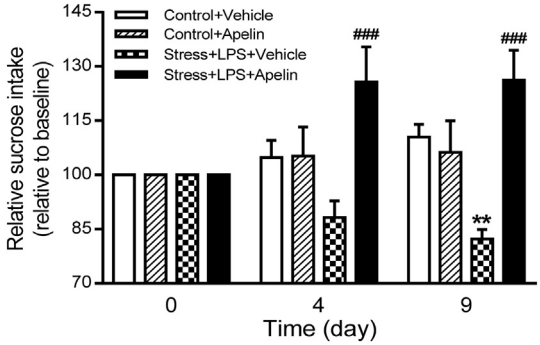

Fig. 3. Effect of Apelin-13 treatment on the relative sucrose intake of rats in the sucrose preference test. **$p < .01$ compared with Control + vehicle rats, ###$p < .001$ compared with Stress + LPS + Vehicle rats. N = 7 per group, all values given are the mean ± SEM.

Finally, we investigated the ratio of the adrenal gland weight to body weight at day 10, following the 9-day variable stressor paradigm (Fig. 2). In stress situations, a synergistic effect of the HPA axis and locus coeruleus / noradrenaline autonomic nervous system take part in the stress response (Chrousos, 1995). Chronic stress can induce excessive activation of the HPA axis and increase corticosterone secretion in rats (Cai et al., 2015). A one-way ANOVA revealed a significant difference in the adrenal weight ratio ($F(3,27) = 23.410$, $P < 0.001$) (Fig. 4). Post hoc comparisons showed that the adrenal weight ratio was dramatically increased in the group co-treated with chronic stress and LPS compared with that in the control group ($p < .001$), and this increase was reversed by apelin-13 treatment ($p < .001$) (Fig. 4).

These findings clearly demonstrate that our stress model has a time-dependent inhibitory impact on depressive-like behavior and apelin treatment could reverse the depression-like behavior of rats.

3.2. Apelin-13 decreases the activation of glial cells in a stress model

We next investigated the effect of chronic stress and LPS co-treatment on the activation of microglia and astrocytes cells in the hippocampus by western blot with antibodies against IBA-1 and GFAP (Fig. 5). A one-way ANOVA revealed that there was a significant difference in the expression of IBA-1 ($F(3,19) = 24.089$, $p < 0.001$) (Fig. 5A) and GFAP ($F(3,19) = 12.120$, $p < 0.001$) between groups (Fig. 5B). Post hoc comparisons showed that the expression levels of IBA-1 and GFAP were dramatically increased (all, $p < .001$) in the group co-treated with chronic stress and LPS compared with the expression in the control group, and this increase was reversed by apelin-13 treatment ($p = .042$ and $p = .018$). These results suggest that chronic stress and LPS co-treatment activates microglial cells, but apelin-13 reverses this trend.

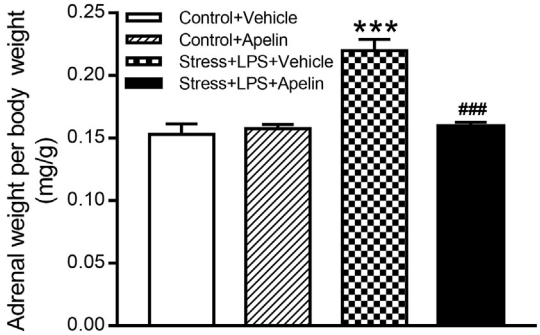

Fig. 4. The adrenal weight ratio. ***$p < .001$ compared with control + Vehicle, ###$p < .001$ compared with Stress + LPS + Vehicle rats. Data represent group mean ± SEM, N = 7 per group.

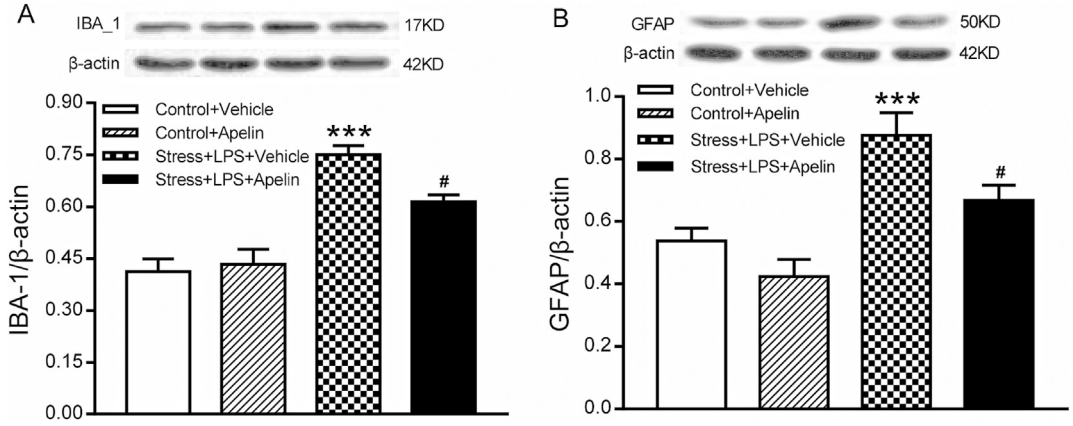

Fig. 5. Effect of Apelin-13 on activation of microglia and astrocytes in rats hippocampus. Representative Western blot illustrating the expression of IBA-1 (A) and GFAP (B) in the hippocampus. Individual samples were normalized to β-actin. ***p < .001 compared with Control + Vehicle rats, #p < .05 compared with Stress + LPS + Vehicle rats. *N* = 5 per group. Data represent group mean ± SEM.

3.3. Apelin-13 decreases the expression of inflammatory mediators in hippocampus of stressed rats

We then investigated whether chronic stress and LPS co-treatment led to the release of inflammatory mediators, such as IL-1β and TNF-α, in the hippocampus. A one-way ANOVA revealed that there was a significant difference in the expression of TNF-α ($F(3,19) = 19.333$, $p < 0.001$) (Fig. 6A) and IL-1β ($F(3,19) = 22.080$, $p < 0.001$) across groups (Fig. 6B). Post hoc comparisons showed that the levels of TNF-α and IL-1β were dramatically increased (all, $p < .001$) in the group co-treated with chronic stress and LPS compared with those in the control group. However, apelin-13 reduced the expression of TNF-α and IL-1β ($p = .012$ and $p = .039$) in the hippocampus. These results suggest that chronic stress and LPS co-treatment increases the release of inflammatory mediators, but apelin-13 reverses this trend.

3.4. Apelin-13 inhibits inflammatory signaling pathways in hippocampus of stressed rats

To examine whether the activation of inflammatory signaling pathways is involved in the effects of apelin-13, which may suppress neuroinflammation in the rat hippocampus after stress exposure, we studied the protein levels of p-NF-κB and p-IKKβ (Fig. 7). A one-way ANOVA revealed that there was a significant difference in the expression of p-NF-κB ($F(3,19) = 35.156$, $p < 0.001$) (Fig. 7A) and p-IKKβ ($F(3,19) = 6.923$, $p = 0.003$) across groups (Fig. 7B). Post hoc comparisons showed that the expression of p-NF-κB and p-IKKβ was significantly higher (all, $p < .001$) in the group co-treated with chronic stress and LPS than in the control group. Conversely, we found that apelin-13 treatment decreased the expression of p-NF-κB and p-IKKβ ($p < .001$ and $p = .025$). These results suggest that chronic stress and LPS co-treatment activates inflammatory signaling pathways, but apelin-13 treatment inhibits the inflammatory signaling pathways in the hippocampus.

4. Discussion

In our present study, we show that chronic stress exacerbated microglial activation after injection of a proinflammatory stimulus such as LPS in the hippocampus, leading to an increase in neuroinflammation mediators in the brain and depressive-like behaviors in rats. However, repeated intracerebroventricular injection of apelin-13 prevented stress-induced microglial overactivation and the expression of cytokines in response to LPS, supporting the idea that apelin-13 has antidepressant and anti-inflammatory activity.

With the development of human society and the spirit of the improvement of living standards, the incidence of depression gradually increased. The data show that depression is currently ranked fourth for disabling mental illness and is predicted to be ranked second in 2030 (Mathers and Loncar, 2006). Animal and human studies have shown that chronic inflammation is frequently associated with the development of depression (Ceretta et al., 2012a, 2012b, 2012c). These studies prompted us to investigate the role of neuroinflammation in the development of depression and the related mechanism to further broaden

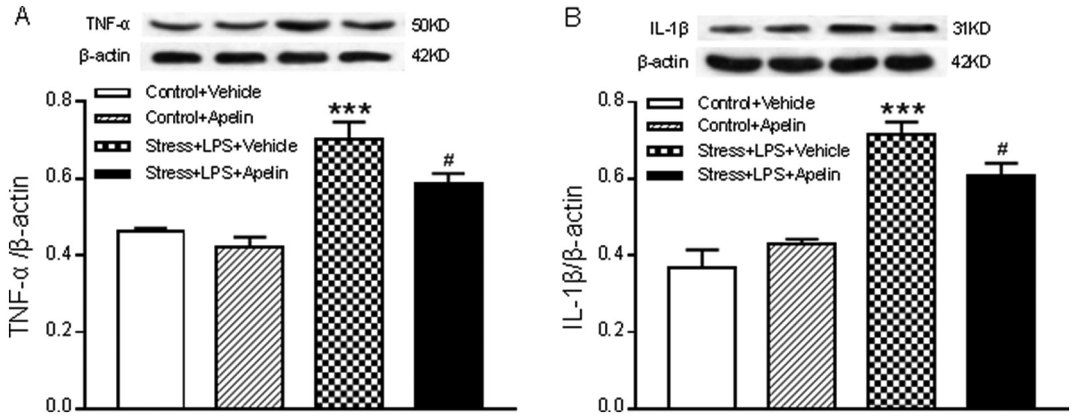

Fig. 6. Effect of Apelin-13 on expression of inflammatory mediators in the hippocampus. Representative Western blot illustrating the expression of TNF-α (A) and IL-1β (B) in the hippocampus. Individual samples were normalized to β-actin. ***p < .001 compared with Control + Vehicle rats, #p < .05 compared with Stress + LPS + Vehicle rats. N = 5 per group. Data represent group mean ± SEM.

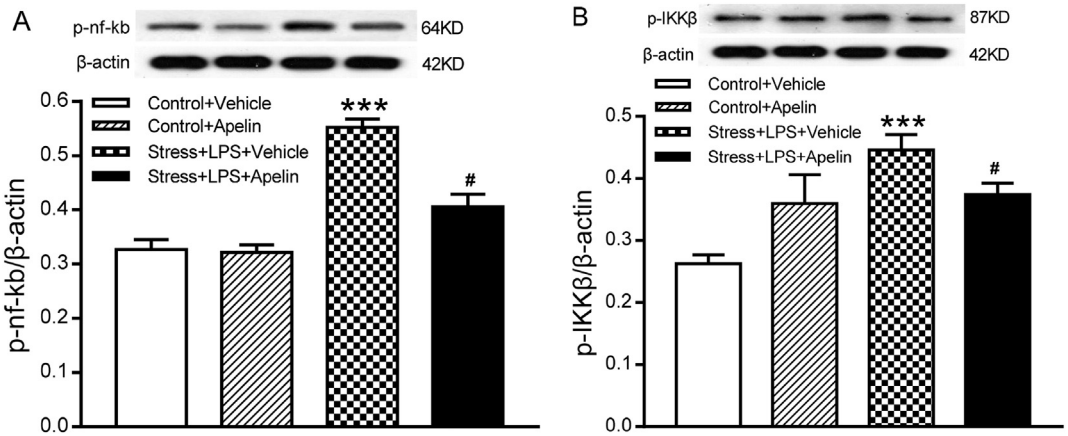

Fig. 7. Effect of Apelin-13 on expression of inflammatory signaling pathways in the hippocampus. Representative Western blot illustrating the expression of p-NF-κB (A) and p-IKKβ (B) in the hippocampus. Individual samples were normalized to β-actin. ***p < .001 compared with Control + Vehicle rats, ###p < .001, #p < .05 compared with Stress + LPS + Vehicle rats. N = 5 per group. Data represent group mean ± SEM.

our understanding of the mechanism of depression. Neuroinflammation may be an important cause of depression. Therefore, we chose to use a depression model to explore the role of neuroinflammation in depression and related mechanisms, as well as whether apelin-13 can change the depressive behavior.

Previous studies have demonstrated the different susceptibility of the CNS to a proinflammatory stimulus induced by a single intraparenchymal injection of LPS (Herrera et al., 2000; Kim et al., 2000). There was no microglial activation in brain areas after 7 days such as the hippocampus to intrahippocampal LPS(Kim et al., 2000), but chronic stress strengthens the sensitivity of hippocampal neurons to a proinflammatory stimulus induced by a single intrahippocampal injection of LPS (Espinosa-Oliva et al., 2011a, 2011b; de Pablos et al., 2006). These studies provide compelling evidence that chronic stress is a key event in activating reactive microglia in the hippocampus when combined with a proinflammatory stimulus (Espinosa-Oliva et al., 2011a, 2011b). We established a depression model using chronic unpredictable stress and intracerebroventricular injection of LPS. The last 9 days of the chronic unpredictable stress included forced swimming, flash, white noise, wet pad, cage tilt and ice-water swimming stress. Stress has been shown to potentially influence systemic inflammatory responses (Cohen et al., 2012), and previous reports have demonstrated an increase in microglial cytokines when LPS was injected in the rat hippocampus (Tanaka et al., 2006).

The hypothalamic-pituitary-adrenal (HPA) axis plays an important role in the survival and growth of the organism, as well as activates and regulates the physiological function of the organism in a stress condition (Calfa et al., 2003). Stress has been shown to be able to induce a decrease in food intake in rats (Gibson-Smith et al., 2015) and the intake of sucrose in the sucrose preference test (Forbes et al., 1996), induce cognitive decline and learning and memory deficits in rats (Clark et al., 2016), and increase the immobility time of rats in a forced swimming test (Sakhaee et al., 2017). Chronic stress can induce glucocorticoid dysfunction and cause negative feedback imbalance of the HPA axis, leading to the occurrence of depression (O'Keane et al., 2012). We used the immobility time of the rat in the forced swimming test, weight gain, adrenal weight ratio, and sucrose preference test to assess depressive-like behavior. The experimental results showed that there was no significant difference in immobility time between the SLV and SLA rats on the first day of the forced swimming test. In addition, after LPS injection and stress, the rats in the SLV group exhibited a significant increase in immobility time. The same trend also appeared in weight gain and sucrose preference test. After the SLV rats were treated with LPS, the weight gain rate decreased significantly, with some rats even showing weight loss. Furthermore, the sucrose preference decreased after chronic stress and LPS injection, proving that our model of depression is of great significance for examining the pathological mechanism of depression. We examined the ratio of the adrenal gland weight to the body weight of rats and found that stress and LPS injection caused an increase in this ratio, suggesting changes in glucocorticoid levels during this stress process, consistent with our previous research.

Glial cells are widely involved in a number of physiological functions in the central nervous system. They provide nutritional support to protect neurons (Stertz et al., 2013), have crucial effects in the anti-inflammatory process, and, under certain pathological conditions, can be rapidly activated. The activation of glial cells includes their proliferation, migration and release of a large number of nerve inflammatory factors. Previous studies have indicated that under normal circumstances, the hippocampus is highly resistant to inflammation, whereas under conditions of stress, LPS can induce a stronger activation of microglia, causing further hippocampal damage. Microglial activation can induce the release of proinflammatory cytokines including IL-1β, IL-6, and TNF-α, induce inflammation attack, synaptic neuron damage and cognitive dysfunction (Réus et al., 2015; Zhao et al., 2019). Several studies have suggested that stress is related to the activation of microglia (Tynan et al., 2013; Couch et al., 2013) and that the abnormal activation of microglia is related to the increase in the production of inflammatory mediators such as IL-1β, IL-6 and TNF-α (Diz-Chaves et al., 2012). Our experiments found that LPS injection in stressed animals resulted in an increase in the expression levels of proinflammatory cytokines such as IL-1β and TNF-α, as shown by western blot. In stressed animals, LPS triggered activation of microglia in the hippocampal formation, thus demonstrating the key role of stress in this event. Our study demonstrated that stress combined with LPS injection can induce microglia and astrocyte activation. Therefore, we suggest that our findings of increased IBA-1 and GFAP expression in rats exposed to stress and LPS treatment compared to those in control rats might be indicative of an adaptive glial response to support degenerating hippocampal neurons, thus providing a new piece of evidence that chronic stress combined with LPS plays a key role in microglia activation in the hippocampal formation.

Nuclear transcription factor-κB (NF-κB) is a class of signaling molecules downstream of TNF-α and plays an important role in the inflammatory response (Blondeau et al., 2001; Wolf et al., 2009). NF-κB is inactive in the cytoplasm due to inhibition by the IκB protein. With the stimulation of inflammation, proinflammatory cytokines can activate the IκB kinase complex to ubiquitinate the IκB protein and then activate NF-κB. NF-κB is then translocated to the nucleus after various modifications where it regulates the expression of inflammatory factors in

combination with the corresponding binding protein (Baeuerle and Baltimore, 1988). We detected the expression of inflammatory factors, such as p-NF-κB and p-IKKβ. Nuclear factor-kappa B (NF-κB) is an important cytokine regulating neuroinflammation. The activation of NF-κB is thought to be part of the stress response as NF-κB is activated by a variety of stimuli and its activation may be associated with astrocyte and microglia activation (Shih et al., 2015; Niranjan, 2013). NF-κB has been known to act as an essential transcription factor for the expression of inflammatory mediators, such as iNOS, COX-2, IL-1β and TNF-α (Lawrence and Fong, 2010), which lead to depressive-like behavior (Biesmans et al., 2013; Yuan et al., 2015). In addition, NF-κB signaling may play a critical role in activation of glia cells (Kim et al., 2014), indicating that the activation of NF-κB signaling precedes microglia and astrocyte activation. Stress, infection and injury can induce the activation of glial cells to release IL-1β, TNF-α and other proinflammatory cytokines and the phosphorylation and direct activation of NF-κB, thereby activating NF-κB nuclear translocation, as well as participate in the expression of inflammatory factors, which lead to the release of a large number of inflammatory cytokines such as IL-1β and TNF-α (Xu et al., 2010; Liu et al., 2015). The various stimuli that activate NF-κB cause phosphorylation of IκB, which is followed by its ubiquitination and subsequent degradation. IκB proteins are phosphorylated by the IκB kinase complex consisting of at least three proteins: IKK1/IKKa, IKK2/IKKβ, and IKK3/IKKg. Activation of NF-κB relies on activation of the IKK complex, in which IKKβ triggers the canonical pathway. Taken together, our results suggest that the expression of p-NF-κB and p-IKKβ is increased in response to stress and LPS treatment, which subsequently leads to the elevation of IL-1β and TNF-α levels, resulting in depression-like behaviors in rats.

The apelinergic system plays a pivotal role in the neuroendocrine response to stress. Apelin and APJ mRNA are highly expressed in the hypothalamus and anterior pituitary gland, key brain areas related to the stress response (O'Carroll et al., 2013). The current study found that apelin has physiological antidepressant, neuroprotective and neurotransmitter regulatory functions (Xin et al., 2015; Han et al., 2014; Telegdy et al., 2013; Yang et al., 2015). In this study, we found that stress and LPS treatment was able to induce depression-like behavior, with a simultaneous increase in the release of inflammatory cytokines. Repeated intracerebroventricular injection of apelin-13 was able to improve the stress and LPS-induced depressive-like behaviors in rats, reduce the release of inflammatory cytokines, and inhibit glial cell activation, suggesting that apelin-13 improved depressive-like behaviors in rats and reduced the release of these factors associated with neurons. In addition, we also found that apelin-13 was able to significantly improve the stress and LPS-induced decrease in sucrose preference (anhedonia) and increase in immobility time in the forced swimming test. In our study, through reducing the release of inflammatory factors, apelin-13 was shown to act as an antidepressant to affect depression-like behavior induced by co-treatment with LPS and chronic stress.

Overall, we found that the rats given a 9-day chronic unpredictable stress and intracerebroventricular injection of LPS showed depression-like behavior. Further study found that stress and LPS co-treatment induced glial cell activation and increased the expression of neuroinflammatory factors. Apelin-13, through its effects on nuclear transcription factor, reduced stress and LPS-induced activation of glial cells and the inflammatory response to alleviate depression-like behaviors in rats, indicating that apelin-13 may be a new type of antidepressant that may act by improving the neuroinflammatory process mediated by NF-κB.

5. Conclusion

Apelin-13 can improve stress and LPS-induced depression-like behavior, which may be mediated by inhibition of the NF-κB-mediated inflammatory response.

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