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Baicalin exerts antidepressant effects through Akt/FOXG1 pathway promoting neuronal differentiation and survival

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ABSTRACT

Background and aims: Impaired neurogenesis in hippocampus may contribute to a variety of neurological diseases, such as Alzheimer's disease and depression. Baicalin (BA), which is mainly isolated from the root *Scutellaria baicalensis Georgi* (traditional Chinese herb), which was reported to facilitate neurogenesis, but how to play the role and the underlying molecular mechanisms are still unknown.

Main methods: In this study, we adopted the chronic unpredictable mild stress (CUMS)-induced mouse model of depression, and then explore antidepressant-like effects and possible molecular mechanisms.

Key findings: We found that BA significantly increased sucrose consumption in sucrose preference test, the number of crossing in open filed test and attenuated immobility time in tail suspension test. Additionally, BA administration notably promoted neuronal differentiation and the number of DCX^+ cells. Moreover, BA facilitated immature neurons develop into mature neurons and their survival. FOXG1, a transcription factor gene, which is crucial for mammalian telencephalon development, specifically stimulates dendrite elongation. BA could reverse the decrease of *p*-Akt, FOXG1 and FGF2 caused by CUMS-induced. Additionally, the expression of FOXG1 and FGF2 significantly decreased when the Akt pathway were inhibited by LY294002 in SH-SY5Y cells. Interestingly, BA failed to counteract the decline.

Significance: These results suggest that BA could promote the differentiation of neurons, which transformation into mature neurons and their survival via the Akt/FOXG1 pathway to exert antidepressant effects.

1. Introduction

Depression, a severe psychiatric disorder, the most obvious symptom is anhedonia [1,2]. Chronic Unpredictable Mild Stress (CUMS) is a well-established animal model of depression, which imitate the clinical core symptom of depression, anhedonia [3]. Although the underlying mechanisms of CUMS-induced depressive-like behaviors are not fully understood, decline of hippocampal neurogenesis is presumed to play a critical role in this mechanism [4]. Adult hippocampal neurogenesis, is a complex and dynamic process, involving the proliferation, differentiation, migration, maturation and survival in NSCs and NPCs [5]. The mainly regions are subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle. Mounting evidences demonstrated that impaired neurogenesis is also linked to depression [6], which can be seriously regulated by lots of environmental and physiological factors, as well as transcription factors, cytokines and neurotrophins [7]. The Forkhead Box transcription factor G1 (FOXG1, BF-1), is critical for the

Abbreviations: AOD, average optical density; BA, Baicalin; BrdU, 5-Bromo-2-deoxyUridine; CUMS, chronic unpredictable mild stress; DCX, Doublecortin; DG, dentate gyrus; FGF2, Fibroblast growth factor; FOXG1, Forkhead Box transcription factor; NeuN, Neuron specific nuclear protein; NPCs, neural progenitor cells; NSCs, neural stem cells; OFT, Open filed test; SGZ, Subgranular zone; SPT, Sucrose preference test; SVZ, Subventricular zone; TLE1, Transducin-like enhancer of split-1; TST, Tail suspension test

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development of the telencephalon, which facilitates its neurogenesis by stimulates dendrite elongation [8]. Overexpression of FOXG1 with lentiviral vectors could inhibit gliogenesis and promote neurogenesis, dramatically stimulates neurite outgrowth [9]. Disrupted FOXG1 in the subpallium impaired cortical interneuron differentiation and tangential migration [10]. It was documented that the inhibition of PKA and Akt kinases in postmitotic neurons, could abolished FOXG1-dependant *P*-CREB1 dendrite elongation and neuritis branching [8]. Wortmannin (a specific inhibitor of PI3K) inhibited Akt activation and attenuated the neuronal differentiation of NSCs induced by valproic acid [11]. It is necessary interact with FOXG1 in mature neurons that TLE1 (Transducin-like enhancer of split-1) exerts promoting neuronal survival. Moreover, TLE1 dependent on the PI3K/Akt pathway [12]. FGFs (fibroblast growth factor) and their receptors influence behavior by affecting brain development. Extensive literature reports that FGF2 treatment reduced depressive-like behaviors, and knockout FGF2 shown reduction in brain volume and hyperactivity, as well as anxiety behaviors [13]. Exogenous FGF2 alleviated the impairment of neurogenesis induced by neuroinflammation [14]. Moreover, perlecan facilitated neural stem cell self-renewal and neurogenesis which mediated FGF2 through Akt and Erk 1/2 pathways [15].

Baicalin (BA, Fig. 1A), one of the predominant flavonoid derivatives, isolated from the dry roots of *Scutellaria baicalensis Georgi,* which has multiple pharmacological effects, including anti-inflammation [16], anti-cancer [17], and anti-apoptosis [18]. Moreover, BA could affect the differentiation of rat NPCs, and transform them into mature neurons in vivo, so which exhibited neuroprotective activity in chronically stress-induced rats [19] and which improved learning and memory impairment after global cerebral ischemia/reperfusion [20]. Recent research shows that BA could also ameliorate neurological function and lessen brain edema in traumatic brain injury model via activating the Akt signal [21]. But the neuro-protective mechanism of BA is still unclear in depression model. In the current of study, we adopted the classical CUMS model of depression to assess the antidepressant effect of BA. Our results revealed that BA alleviated depressive-like behaviors in CUMS model. The pathogenesis of depression is more complicated and unclear, but adult hippocampal neurogenesis plays an important role in the treatment of depression. The effect of Baicalin on neurogenesis was further investigated by immunofluorescence assay, and the molecular mechanism was explored by the inhibitor (LY294002) of PI3K in SH-SY5Y cells.

2. Materials and methods

2.1. Animals

Male ICR mice (23-26 g), were purchased from the Experimental Animal Center in Jiangsu Province (Nanjing, China). All animals were housed in cages under standardized environmental conditions (12 h light/dark cycle, 25 ± 2 °C) with free access to food and water. All animals' experiments were performed in strict accordance to the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province.

2.2. Drugs and reagents

Baicalin (BA, purity ≥ 98%) was purchased from Nanjing Jingzhu Biological Engineering Co, Ltd. (Nanjing, China). 5-Bromo-2-deoxyUridine (BrdU) was obtained from Sigma-Aldrich Co (St Louis, USA). Fluoxetine hydrochloride (Flu) was brought from Changzhou Siyao Pharmaceuticals Co, Ltd. (Changzhou, China). LY294002 (purity ≥ 98%) was acquired from the Apexbio (Boston, MA, USA).

2.3. Experiment design

Forty-eight mice were randomly divided into four groups and were tested for the behavior (n = 8–10): Control group (saline), CUMS model group (saline), CUMS + Flu (15 mg/kg), CUMS + BA (60 mg/kg). The CUMS procedure was executed, as previously reported with minor modifications [9]. Briefly, the entire experiment lasted for six weeks, which CUMS groups were exposed to stressors for 3 weeks, as shown in Table 1, and then BrdU (150 mg/kg, i.p) for 3 consecutive days and drug treatment for the last 3 weeks at the same time. The Flu and BA groups received administration via i.g. After 6 weeks, mice underwent behavioral evaluation. BA was dissolved in DMSO and diluted 100 times with PBS to a final concentration of 0.045 mg/ml in vitro. The experimental schedule was in Fig. 1B.

2.4. Behavior analysis

2.4.1. Sucrose preference test (SPT)

SPT was used to evaluate CUMS-induced anhedonic-like behavior. The mice were pre-exposure to 1% sucrose after 12 h food and water deprivation [21]. Mice were kept in single cages following 12 h of water and food deprivation in the dark phase, and were individually given two bottles, 1% sucrose solution and tap water. The bottles were weighted to evaluate water consumption after 12 h. Sucrose preference (%) = [sucrose consumption/ (sucrose consumption + water consumption)]*100%.

2.4.2. Open field test (OFT)

To assess the effects of BA on locomotors behavior in mice, and OFT was performed as described previously [22]. The mice were individually placed in the center area of cage, and lasts 6 min with a quiet environment. The number of crossing with all paws (crossings) of the

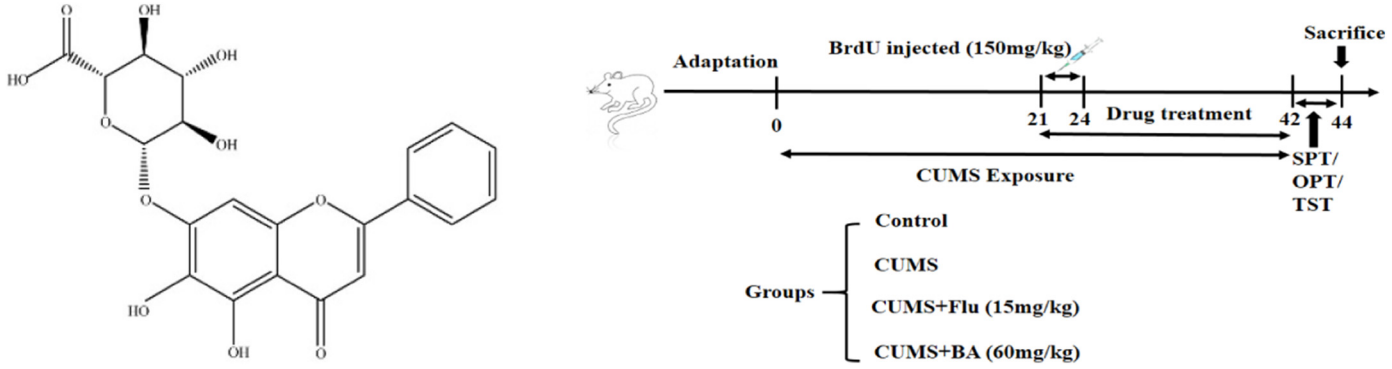

Fig 1. a The structure formula of BA.
b Timeline, groups and schematic representation of the experimental procedure for chronic unpredictable mild stress (CUMS) and treatments. BA, Baicalin; SPT, sucrose preference test; OFT, open field test; TST, tail suspension test.

Table 1
Nine different stressors of CUMS model.

Stressor	Duration
Clip tail	5 min
Horizontal oscillation	5 min
Noise	5 min
Physical restraint	5 min
Crowding	12 h
Fasting	12 h
Water deprivation	12 h
Overnight illumination	12 h
Tilted cage (45°)	12 h

mouse was counted in the last 4 min.

2.4.3. Tail suspension test (TST)

Immobility time of mice was evaluated by the TST as the method previously described by our laboratory [23]. Briefly, the tail of mice was suspended on the stand during the 6 min, and the immobility time was recorded at the final 4 min. Mice were considered immobile when they hung passively without struggling.

2.5. Western blotting

The mice hippocampal were quickly isolated and then frozen at −80 °C for protein analysis. Western blotting was performed as described previously [24]. Quantified using the bicinchoninic acid assay kit. An equivalent amount of protein was transferred to PVDF membranes, which were blocked with 5% skimmed milk at room temperature for 2 h. and the membranes were then incubated the primary antibodies [anti-Akt (WL01619), anti-phospho-Akt (WLP001a), anti-FOXG1 (ab18259) and anti-FGF2 (ab92337)] 4 °C overnight. After 3 times washes, the membranes were exposed to secondary antibody during 2 h at room temperature. Imaging system (Bio-Rad, Hercules, CA, USA) was used measured density of bands and quantified analysis through image J software.

2.6. Immunofluorescence

Mice were deeply euthanized and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA) in PBS. The whole brains were quickly dehydration with 10%, 20%, 30% sucrose solution [25]. And then the brains were cut into 30 μm thick by means of frozen sectioning. For BrdU staining, sections were incubated in 1 N-HCl for 10 min at ice, 2 N-HCl for 20 min at room temperature, then at 37 °C water bath for 10 min, and finally neutralized with 0.1 M boric acid buffer solution (pH = 8.5). After sections were blocked with 10% goat serum for 2 h, the primary antibody BrdU (ab136650) and NeuN (ab177484), DCX (ab18723) were incubated at 4 °C overnight. The sections were double-immunolabeled with BrdU/NeuN (cell survival analysis), and DCX (cell differentiation). Finally, the sections were incubated in dark with appropriate Alexa fluor-488/594-conjugated secondary antibodies (ab150077, ab150115), and photographed by fluorescence microscopy. Image J software was used to analyze optical density.

2.7. Cell culture

The human neuroblastoma cell lines (SH-SY5Y) were cultured in DMEM/F12 medium, and 10% fetal bovine serum and 1% penicillin /streptomycin were added. MTT analysis was performed as described previously [26]. When the cells had grown to 80% confluence, which were seeded in six well plates, seed 2×10^5 cells per well in 2 ml normal growth medium, which divided into four groups: Control, Control + BA, LY294002 (10 μM), LY294002 + BA (100 μM). The expression of FOXG1 and FGF2 were evaluated by western blotting.

2.8. Statistical analysis

Data were expressed as the mean ± SEM and analyzed by one way ANOVA followed by Turkey's test. The value ($p < 0.05$) was considered statistically significant. Data analysis was performed using GraphPad Prism 5.0 and Image J software.

3. Results

3.1. BA reversed the depressive-like behavior of CUMS-induced mice

3.1.1. The sucrose preference test (SPT)

As shown in Fig. 2A, an obviously difference was presented between groups in the SPT. Compared with the vehicle-treated group, the sucrose intake was obviously reduced in CUMS exposure. On the contrary, BA ($P < 0.01$) and Flu ($P < 0.05$) administration was significantly increased the sucrose consumption [F(3,36) = 4.498], so ameliorated the anhedonia behavior of the mice.

3.1.2. The open filed test (OFT)

The number of entries in the center of the open filed arena was used to assess spontaneous activity of mice. As shown in Fig. 2B, the number of crossing in CUMS groups was significantly lessen compared to vehicle-treated group [$P < 0.01$, F (3, 36) = 7.386]. Additionally, the CUMS + BA60 group, in comparison with the CUMS + Flu15 group, had significantly the more number of crossing ($P < 0.01$) over 4 min intervals.

3.1.3. The tail suspension test (TST)

The effects of BA on desperate behaviors were evaluated through TST. The comparison of the Control group and CUMS groups were shown in Fig. 2C, the CUMS exposure clearly caused increase in immobile durations of mice for the TST test. In contrast, BA (60 mg/kg)

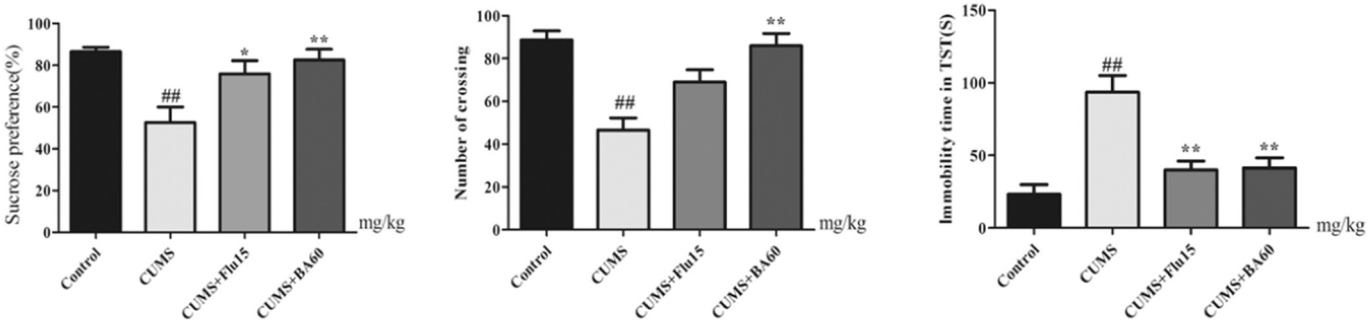

Fig. 2. Effects of BA on depressive-like behaviors in CUMS-induced mice. a Sucrose preference test (SPT). b Open field test (OFT). c Tail suspension test (TST). Values are shown as the mean ± S.E.M ($n = 10$). #P<0.05 and ##P<0.01 vs control group,*p<0.05 and **p<0.01 vs CUMS-induced groups.

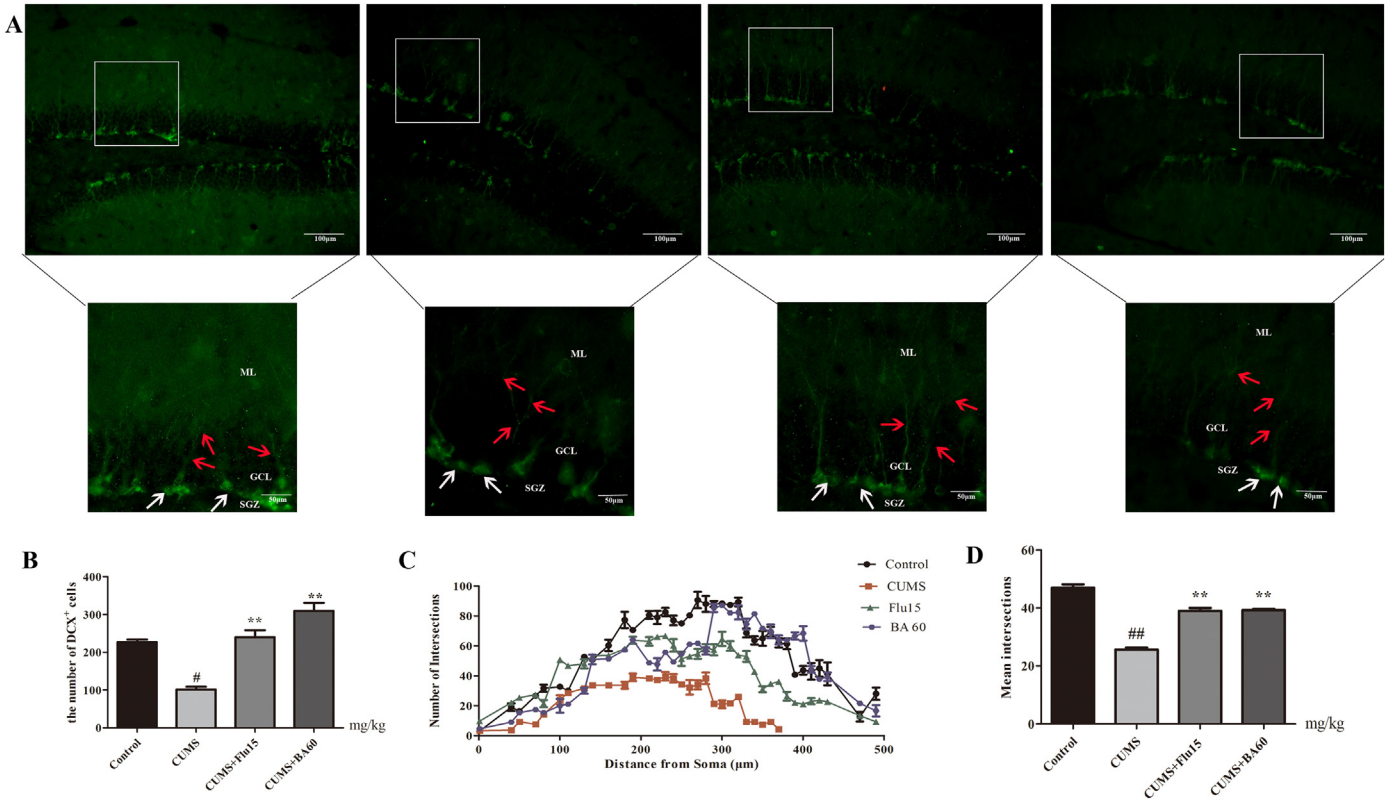

Fig. 3. Effects of BA on the differentiation of immature neurons in hippocampus dentate gyrus. a Representative photomicrograph shown immunostaining of DCX (a marker of immature neurons; red). Scale bar 100 μm and 50 μm. White arrows indicated the cell bodies (soma) of immature neurons located in the SGZ, red arrows indicated the dendrites present in the GCL and molecular layer (ML). b The quantification of positive cells DCX in the GCL. c The number of dendritic intersections of neurons in the DG by Sholl analysis. d The number of mean number of intersections as quantified by Sholl analysis. Values are shown as the mean ± S.E.M ($n = 3$) #p<0.05 and ##p<0.01 vs control group,*p<0.05 and**p<0.01 vs CUMS-induced groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Flu (15 mg/kg) treatment, in comparison with the CUMS group, had greatly shortened immobility time [$P < 0.01$, F (3, 36) = 16.288], alleviated the despair behavior of the model mice.

3.2. BA modulated dendritic morphology and hippocampal neurogenesis

Initially, we examined the effect of BA on immature granule neurons in the SGZ (Fig. 3A-D) by immunostaining of DCX, which is a marker of newborn neurons. The CUMS group, in comparison with the other groups, had greatly declined the number of DCX^+ cells present along the entire SGZ of the hippocampal DG (Fig. 3B). Quantitative assessment the number of DCX intersections by Sholl analysis [27]. Compared with the vehicle-treated counterparts, CUMS resulted in reduction of the number of intersections (Fig. 3C-D, $P < 0.01$). Interestingly, further analysis revealed that a significant increase in the number of intersections along the dendrites of DCX-positive cells only occurred in animals treated with either Flu or BA ($P < 0.01$), suggesting that BA could modulate dendritic morphology of newborn neurons, promoting differentiation of NSC in CUMS-induced mice.

Further, we evaluated whether the effect of BA treatment on the maturation and long-time survival of NSC. We administered BrdU (150 mg/kg, i.p.) for 3 consecutive days and mice were killed at 21 day. We found that the AOD of $BrdU^+/NeuN^+$ was significantly reduced in the DG of CUMS-induced mice Fig. 4A-B, as compared with control ($P < 0.01$). However, BA treatment greatly reversed the reduction ($P < 0.01$) present along the entire SGZ of the hippocampal DG, suggesting that promoting of neurogenesis could enhance the maturation and survival of NSC with BA treatment.

3.3. BA up-regulated Akt/FOXG1 pathway in the hippocampus of depressive-like mice

To assess the underlying mechanism of BA in treatment of depression, we tested the levels of related proteins in the hippocampus of the mice. As shown in Fig. 5, we found that the expression levels of *p*-Akt/Akt ($P < 0.05$), FOXG1 ($P < 0.05$) and FGF2 ($P < 0.01$) were significantly reduced after CUMS exposure. In contrast, chronic treatment of BA (60 mg/kg, $P < 0.01$, $P < 0.05$, $P < 0.05$) reversed the attenuation with the consistent effect of Flu. Thence, these results suggested that BA could modulate the Akt/FOXG1 pathway to exert antidepressant effect.

3.4. BA failed to regulation Akt/FOXG1 pathway when the Akt pathway were inhibited by LY294002

To further elucidate the effect of BA on the Akt/FOXG1 pathway, we performed LY294002 to treat SH-SY5Y cells. Interestingly, the expression of FOXG1 and FGF2 was consistent between the exposed LY294002 group and BA group, which was not improved the decline of proteins by the chronic treatment of baicalin (Fig. 6, $P < 0.01$). Taken together, these results suggested that BA regulated Akt/FOXG1/FGF2 pathway to treatment of depression.

4. Discussion

Baicalin (BA), a major flavonoids compound isolated from the extracts of *Scutellaria Radix*. In our previous work, the *Scutellaria Radix* had been reported to improve the process of neurogenesis and ameliorate depressive-like behaviors, and the main of pharmacodynamics

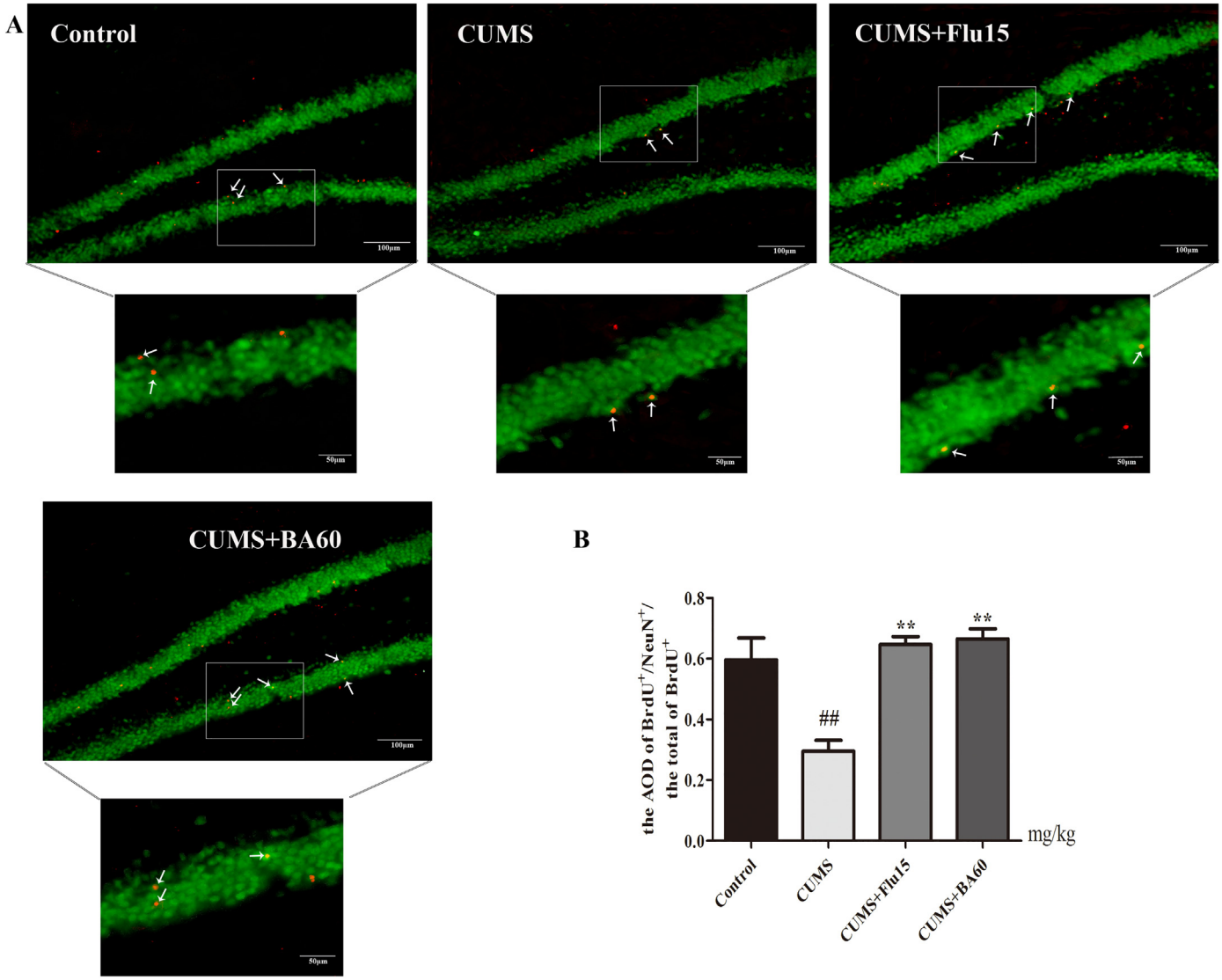

Fig. 4. Effects of BA on the maturation and survival of immature neurons in hippocampus dentate gyrus. a Representative inverted microscope images shown immunostaining of NeuN (a marker of mature neurons; green) and BrdU (a cell proliferation marker; red) in the GCL of hippocampal DG. Scale bar 100 μm and 50 μm. b Proportion of $BrdU^+/NeuN^+$ cells in all $BrdU^+$ cells. Values are shown as the mean ± S.E.M ($n = 3$) # $p < 0.05$ and ## $p < 0.01$ versus control group,*$p < 0.05$ and **$p < 0.01$ versus CUMS-induced groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

basis is BA [28]. Additionally, it had been confirmed that BA possessed efficient antidepressant effects, which was associated with inhibiting of the neuroinflammatory in the brain of mice [29]. The neuroprotective effects of BA have also been confirmed in several central nervous system (CNS) diseases. Hence, we used chronic unpredictable mild stress animal model, to evaluate the antidepressant effects of baicalin, and then further determine underlying mechanism. Interestingly, BA could reverse the disorder behaviors confirmed by SPT, OFT and TST in CUMS-induced mice. Simultaneously, BA could also stimulate hippocampal neurogenesis through enhancing the expression of DCX^+ in immature neurons and then developing maturation and survival. Moreover, we found that BA could exert the role of antidepressant effects via regulation the expression of *p*-Akt and its downstream proteins.

Considerable evidence has demonstrated that anhedonia is clinical core symptom of depression, whereas CUMS-induced behavioral changes could mimic the symptoms of human depression [3,30], which is an indispensable model of depression. The CUMS-induced model mice were used to evaluate the effect of antidepressant and potential mechanism of BA. In this study, long-term exposure of experimental mice developed depressive-like behaviors by the accession the SPT, OFT and TST, which were in line with the previous studies [31,32]. All of these behavioral phenotypes are indications of an enhanced state of depressive-like behavior. Compared with stressed group, our study demonstrated that the anhedonia-like behaviors in sucrose preference test were enhanced and despair behaviors in tail suspension test were shorten with chronic treatment of BA. On the other aspect, the open filed test was frequently employed to identify the emotional profile of mice, included locomotors activity, exploratory behavior in depressed rodents. We found that BA increased the number of crossing, in comparison with model group. Thence, the chronic treatment of BA could ameliorate the depressive-like behaviors in CUMS-induced mice.

Depression is considered as cognitive and affective disorder [33]. In a number of reports, impaired neurogenesis is strongly associated with the disorders, increased susceptibility to stress-induced depressive behaviors. Current clinical evidence indicates the relationship between the abnormal hippocampal structures and depression, which found the smaller hippocampal volume in the patients with depression [34],

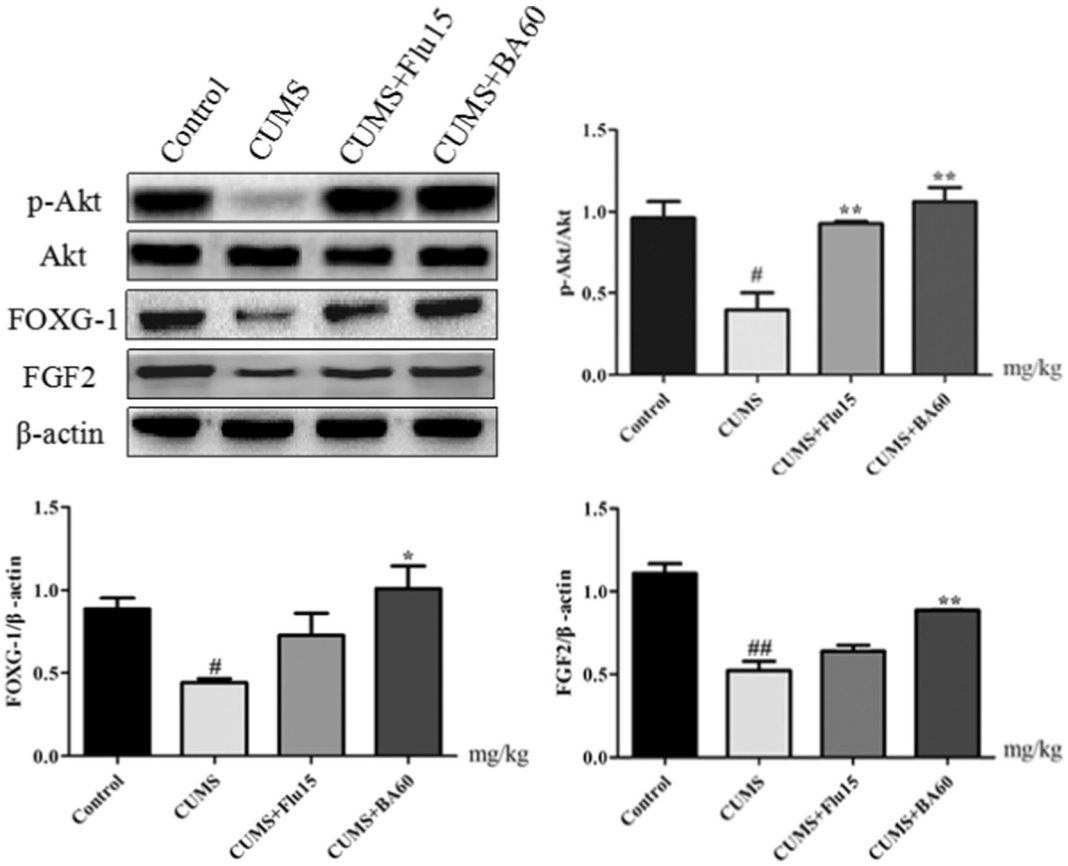

Fig. 5. Effects of BA on the expression of Akt/FOXG1 pathway-related proteins in the hippocampal a Expression of related proteins. b Relative levels of phospho-Akt. c Relative levels of FOXG1. d Relative levels of FGF2. Date were expressed as mean ± S.E.M ($n = 3$). # $p < 0.05$ and ## $p < 0.01$ versus control group,*p < 0.05 and **p < 0.01 versus CUMS-induced groups.

compared with healthy individuals, which may be associated with dendritic retraction, cellular shrinkage and reduced neurogenesis [35]. Thence, we further explore the relationship between depression and neurogenesis, and found that CUMS caused the absence of neurogenesis in depression mice. However, the changes were reversed by BA treatment through increasing the number of dendritic intersections, and number of DCX^+ immature neurons in the hippocampal DG. In addition, chronic treatment of BA, in comparison with the CUMS group, significantly increased the expression level of $BrdU^+/NeuN^+$ co-labeled in the DG. Sum up, these results illustrated that BA treatment increased the adult neurogenesis in hippocampus. Restoring hippocampal neurogenesis is expected to be the basis for clinical treatment of depression.

Akt signal is involved in the regulation of a variety of neurological diseases. More recent findings showed that active Akt could improve the diabetic cognitive in the hippocampus of Type 2 diabetes mellitus mice [36]. Moreover, inhibition of PI3K/Akt signaling reduced neurogenesis in NPCs, whereas constitutive activation of Akt or phosphatase could reserve the change [37]. Additionally, it has been reported that Akt activation caused expression of learning-related proteins, improved cognitive functions in AD model, which may become potential mechanism for treatment AD [38]. In the present investigation, attenuation of Akt and *p*-Akt by CUMS was inhibited by chronic treatment of BA, which was in line with the previous reports [39]. Very interestingly, the above change accompanied with the upward adjustment of FOXG1 expression in the treatment groups. FOXG1 (formerly called BF-1), is a unique transcription factor in the forkhead box protein family, which interacts with FOXO/SMAD and activates TGF-β and PI3K/Akt signaling, participate in cell activities [40]. Additionally, in vitro in embryonic cortical progenitors, FOXG1 inhibits gliogenesis and promote neurogenesis, dramatically stimulates neuritis outgrowth [9]. Moreover, FOXG1 overwhelmed the active of dendritogenesis antagonizers in immature neurons, which reversed the decline of later dendritogenic effectors downregulates and ultimately resulting in dendrite elongation [8]. Unexpectedly, there are reports demonstrated FOXG1 has been determined a new and important function in mature neurons, which could facilitate survive of neuron, prevent neuronal death [41]. In Rett syndrome, enhanced FOXG1 expression inhibited methyl-CpG binding protein 2 (MeCP2) neurotoxicity though Akt activation, which exerts neuroprotection [42]. In our study, treatment with BA elevated the expression of FOXG1 by the activation of Akt. These results demonstrated that BA promotes neurogenesis through Akt/FOXG1 pathway.

Fibroblast growth factor (FGF2, also known as basic FGF), was reported the expression decreases in the hippocampal and frontal cortex of depressed patients [43], which was consistent with CUMS-induced mice. In the adult subventricular zone, FGF-2 signaling plays an important role in promoting neuronal stem cell self-renewal and neurogenesis [15]. Moreover, FGF2 exerts critical role in determining progenitor cell biology during early striatum development in Huntington's disease [45]. In this study, we found that treatment of BA reversed the descendant of FOXG1 and FGF2 in model mice. However, BA failed to reverse the decline of FOXG1 and FGF2 in SH-SY5Y cells, when the Akt pathway was inhibited by LY294002. Whereby, these data highlighted that BA exerts the mechanism of antidepressant via Akt/FOXG1/FGF2 pathway in the CUMS exposure mice.

5. Conclusion

Based on these considerations, our results illustrated that the treatment of BA ameliorated the depressive-like behaviors in CUMS-

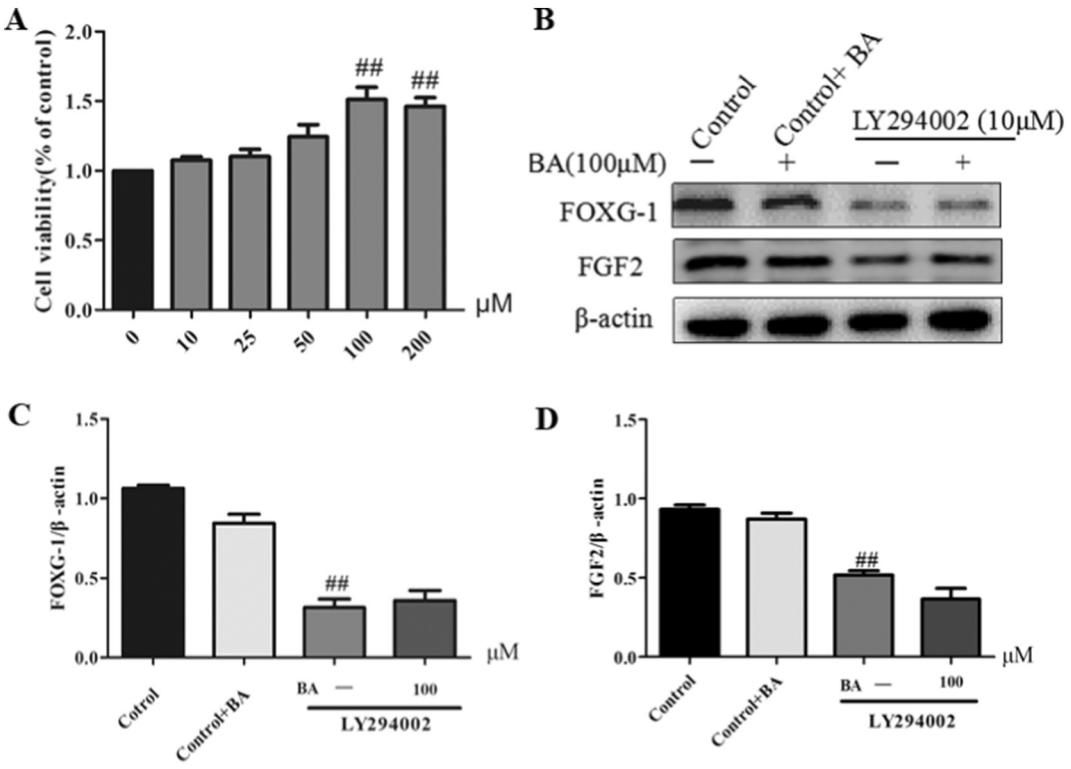

Fig. 6. Effects of BA on the expression of FGF2 when the Akt signal were inhibited by LY294002 in SH-SY5Y cells. a The MTT analysis of BA. b Expression of related proteins. c Relative levels of FOXG1. d Relative levels of FGF2. Date were expressed as mean ± S.E.M (n = 3). #p<0.05 and ##p<0,01 vs control group,*p<0,05 and **p<0.01 vs LY294002 groups.

induced mice. Moreover, we found that adult hippocampal neurogenesis plays a critical role in antidepressant. BA could act on multiple stages of neurogenesis, including the differentiation of immature neurons, which then transform into mature neurons and promote its survival, and the effect could be mediated by the Akt/FOXG1/FGF2 axis. Overall, our study shown that BA, as a potential drug candidate, regulated neurogenesis-associated pathway, which is a viable therapeutic approach to depression.

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Disclosure

The authors have declared no conflicts of interest.

Author contribution

Ruyi Zhang designed this research; Ruyi Zhang, Yawei Li, Dongni Liu, Lixing Xu and Kaili Liu conducted the animal experiments; Zhongxuan Ma wrote the manuscript; Xueyang Deng and Rong Qu instructed the experiment. Zhanqiang Ma and Shiping Ma revised the manuscript.

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