Baicalin influences the dendritic morphology of newborn neurons in the hippocampus of chronically stressed rats

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Abstract
Chronic stress models, established in adult Sprague-Dawley rats through a 14-day subcutaneous injection of 40 mg/kg corticosterone, once per day, were given a daily oral feeding of 50 mg/kg baicalin. The study was an attempt to observe the effect of baicalin on neurogenesis in chronically stressed rats. Results showed that subcutaneous injection of corticosterone significantly decreased the total number of doublecortin-positive neurons in the hippocampus. The reduced cell number caused by corticosterone was mainly due to the decrease of class II doublecortin-positive neurons, but the class I doublecortin-positive neurons were unaffected. Baicalin treatment increased the number of both class I and class II doublecortin-positive neurons. In addition, doublecortin-positive neurons showed less complexity in dendritic morphology after corticosterone injection, and this change was totally reversed by baicalin treatment. These findings suggest that baicalin exhibits a beneficial effect on adult neurogenesis.

Key Words
neural regeneration; traditional Chinese medicine; neurogenesis; neurodegenerative disease; baicalin; stress; hippocampus; neurons; doublecortin; dendrites; cognition; mood regulation; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) The influence of baicalin on the differentiation and maturation of rat neural precursor cells into neurons was investigated, using in vivo experimental evidence to show that baicalin promotes neurogenesis.
(2) Using classification methods, the effect of baicalin on the number and morphology of class I and class II doublecortin-positive newborn neurons was observed.
(3) Baicalin promoted the proliferation and maturation of neural precursor cells, and exhibited neuroprotective activity in chronically stressed rats.
(4) Baicalin appears to have therapeutic actions and clinical applications for the improvement of cognitive function and emotional regulation.

INTRODUCTION
Adult neurogenesis is a dynamic process that occurs in the dentate gyrus and subventricular zone in mammals. This life-long process has been regarded to be closely related with the occurrence of psychiatric disorders and cognitive functions[1].

Adult neurogenesis in the dentate gyrus is vulnerable to various challenges, including inhibitory and stimulating challenges[2-5]. One
inhibitory challenge is to induce chronic hypercortisolemia in experimental animals by the daily injection[6] or oral administration of corticosterone[7]. Numerous studies have shown that chronic treatment of high-dose corticosterone impairs adult neurogenesis in the dentate gyrus[8-10], and damaged neurogenesis is consequently associated with behavioral deficits, including depression-like behaviors[11-12], reduced learning and memory[13], and impaired sexual activities[14]. The significance of adult neurogenesis and effective ways to prevent corticosterone toxicity of adult neurogenesis need to be explored.

Baicalin, a commercially used Chinese herbal medicine, is a flavonoid isolated from the root of Scutellaria baicalensis Georgi[15]. As shown in Figure 1, purified baicalin, with a molecular weight of 447.092 Da (C_{21}H_{18}O_{11}), is known to be capable of effectively passing through the blood-brain barrier and the gastrointestinal tract[16].

![Figure 1 Structure of baicalin.](image)

Its anti-inflammatory activities have been well recognized in different infectious models, including pancreatitis[17-19], hepatitis[20], and autoimmune encephalomyelitis[21]. Furthermore, it has been reported that baicalin promotes the neuronal differentiation of several cell lines in vitro, including human umbilical cord blood mesenchymal stem cells[22], rat bone marrow stromal cells[23], and neural progenitor cells[24]. However, little is known if baicalin has any effects on adult neurogenesis in vivo. Adult neurogenesis consists of multiple steps: proliferation, differentiation, migration, maturation and synaptic integration[25]. Briefly, young neurons are generated from the progenitor cell pool in the proliferation stage and differentiation stage; a small number of young neurons migrate to their target region and acquire mature neuronal features, and eventually they integrate into the existing neuronal circuits. Doublecortin, a protein promoting microtubule polymerization, is present in migrating neuroblasts and young neurons[26-27], and is barely expressed in non-neurogenic regions in the mature central nervous system[28]. During adult neurogenesis, expression of doublecortin starts as neuroblasts are generated, peaks in the 21st week, and is downregulated concomitantly with the appearance of the mature neuronal marker NeuN[29-30]. Therefore, doublecortin can be used as a long-term marker in adult neurogenesis. Immunohistochemical staining of doublecortin has been used as a powerful tool in basic research to study the positive[31-33], as well as negative modulators[34-35] of the number and morphology of newly formed neurons in adult neurogenesis.

The aim of this study was to investigate the protective effects of baicalin on adult neurogenesis in the dentate gyrus of chronically stressed rats, using the immunohistochemical staining of doublecortin.

**RESULTS**

**Quantitative analysis of experimental animals**

Twelve adult rats were randomly divided into four groups and subjected to the following treatments: (1) PBS + vehicle group (14-day oral feeding with PBS plus vehicle injection); (2) PBS + corticosterone group (14-day oral feeding with PBS plus 40 mg/kg corticosterone injection); (3) baicalin + vehicle group (14-day oral feeding with 50 mg/kg baicalin plus vehicle injection); (4) baicalin + corticosterone group (14-day oral feeding with 50 mg/kg baicalin plus 40 mg/kg corticosterone injection). There were three rats in each group. All rats were involved in the final analysis.

**Baicalin significantly increased the number of doublecortin-positive cells in the hippocampus of corticosterone-treated chronically stressed rats**

To study the effects of baicalin on adult neurogenesis, we checked the immunoreactivity of doublecortin in the hippocampus, a well-known protein expressed in newly formed neurons, which is associated with migration and differentiation of neuronal progenitor cells[36]. As shown in Figure 2A, most of the doublecortin-positive cell bodies were located at or just beneath the bottom of the granular cell layer, with short or long processes. First, the total population of doublecortin-positive neurons was quantified by Stereo Investigator software. Two-way analysis of variance revealed a significant effect of corticosterone ($F_{[1, 11]} = 174.828, P < 0.001$) and an interactive effect of corticosterone and baicalin ($F_{[1, 11]} = 198.20, P < 0.001$) on the total number of doublecortin-positive cells (Figures 2A, C). Corticosterone injection significantly reduced the total number of doublecortin-positive cells ($P < 0.001$). This effect was reversed by baicalin treatment ($P < 0.001$; Figure 2A, C).
To further address which subset of the doublecortin-positive cells was affected, i.e. the relatively younger or older cells, we counted class I and II doublecortin-positive cells (Figure 2B). As shown in Figure 2B, class I doublecortin-positive cells only had very short horizontal processes, without a long process extending through the granular cell layer. On the contrary, class II doublecortin-positive cells displayed a long vertical process branching through the granular cell layer. Almost no effect of corticosterone injection was found on the number of class I cells ($P > 0.05$; Figure 2D), and no effect of baicalin treatment alone was found on the number of class I cells ($P > 0.05$; Figure 2D). However, there was a significant interactive effect of corticosterone and baicalin treatment ($P < 0.001$) on class I doublecortin-positive cells. Baicalin treatment in corticosterone-treated rats displayed a higher number of class I doublecortin-positive cells than the rats in the PBS + vehicle group (control, $P < 0.001$; Figure 2D).

Two-way analysis of variance revealed that corticosterone significantly reduced the number of class II doublecortin-positive cells ($F_{(1, 11)} = 54.624, P < 0.001$; Figure 2E). Baicalin treatment alone had no effect in control rats ($P = 0.273$). However, baicalin treatment significantly increased the number of class II doublecortin-positive cells in the corticosterone-treated chronically stressed rats, but the increased number was still lower than the control level. The above findings demonstrated that reduced neurogenesis in the hippocampus caused by corticosterone injection might be mainly due to the reduced number of class II doublecortin-positive cells, whereas class I doublecortin-positive cells were slightly affected, and baicalin treatment restored this reduction in neurogenesis by increasing the number of both class I and class II doublecortin-positive cells.

Baicalin restored the morphological changes of doublecortin-positive cells in the hippocampus of corticosterone-treated chronically stressed rats

Dendritic morphology is regarded as one of the important indicators in neuronal maturation[37-38]. In the present study, we adopted dendritic length and the number of intersections in Sholl analysis[39] to study the complexity of dendritic morphology in doublecortin-positive cells (Figure 3A). In brief, the outline of one single neuron was traced.
Figure 3  Effect of baicalin treatment on dendritic tree of doublecortin-positive cells in the dentate gyrus of corticosterone (CORT)-treated rats.

(A) Representative image (left) and traces by Filament tracer software of doublecortin-positive cells in the dentate gyrus.

(B) PBS and baicalin-treated rats displayed similar dendritic trees; CORT-treated rats displayed a less complex morphology of dendritic tree, while baicalin treatment was able to restore the morphological damage caused by CORT.

(C, D) The dendritic length (C) and the number of intersections (D) were analyzed in different groups. Both the number of intersections and dendritic length represent the complexity of the dendrite.

(E, F) Quantitative analysis of dendritic length (E) and the number of intersections (F) in different concentric circles.

Data are expressed as mean ± SEM; there are three rats in each group. Three cells in the granular cell layer from each animal were analyzed, under a 40 × lens of a light microscope. aP < 0.05, vs. PBS + vehicle (Veh) group; bP < 0.05, vs. PBS + CORT group by two-way analysis of variance followed by Student-Newman-Keuls test.
The outlined dendritic tree was analyzed using a set of concentric circles (diameter 10–200 µm) centered at the centroid of the cell body. The intersections with the concentric circles and the dendritic length covered by every two concentric circles were counted, respectively. It was found that 40 mg/kg corticosterone injection dramatically reduced the dendritic length (PBS + corticosterone vs. PBS + vehicle: 80 µm $P = 0.025$, 90 µm $P = 0.006$, 100 µm $P = 0.001$, 110 µm $P = 0.004$; Figures 3C, E) and the number of intersections (PBS + corticosterone vs. PBS + vehicle: 70 µm $P = 0.01$, 80 µm $P = 0.04$, 90 µm $P = 0.004$; Figures 3D, F), two parameters that reflect the complexity of dendritic trees in neurons. However, after 50 mg/kg baicalin treatment, the dendritic length of doublecortin-positive cells (baicalin + corticosterone vs. PBS + corticosterone: 80 µm $P = 0.014$, 90 µm $P = 0.024$, 100 µm $P = 0.009$, 110 µm $P = 0.05$; Figures 3C, E), as well as the number of intersections in corticosterone-treated rats significantly increased (baicalin + corticosterone vs. PBS + corticosterone: 70 µm $P = 0.138$, 80 µm $P = 0.035$, 90 µm $P = 0.018$; Figures 3D, F). No obvious changes in dendritic morphology were observed after baicalin treatment alone (Figures 3B–F). These findings showed that baicalin treatment was able to maintain the dendritic complexity of newborn neurons subjected to 40 mg/kg corticosterone challenge.

**DISCUSSION**

In the present study, baicalin treatment enhanced neurogenesis by increasing the number of both class I and class II doublecortin-positive cells in the hippocampus of corticosterone-induced chronically stressed rats. Furthermore, baicalin treatment restored the morphological complexity of doublecortin-positive cells in the corticosterone-treated rats. The neuroprotective effect of baicalin agreed with previous findings\[^{22-24,40-41}\]. Several studies have shown that baicalin has a powerful ability to promote neuronal differentiation in different lines of stem cells, including mouse C17.2 neural stem cells\[^{40}\] and human mesenchymal stem cells\[^{41}\]. However, one limitation in all the studies was that those experiments just provided \textit{in vitro} evidence showing the beneficial effects of baicalin. To our knowledge, the present data provided the first line of \textit{in vivo} evidence that baicalin can exhibit a protective effect on adult neural progenitor cells.

During adulthood in mammals, new neurons are continuously produced from a pool of neural progenitor cells located in two brain regions: the dentate gyrus and the subventricular zone. Then, a small amount of newly formed neurons manage to mature and integrate into the existing neuronal circuits. The whole process is called “adult neurogenesis”, consisting of several stages: proliferation, differentiation, migration, maturation, and synaptic integration. Adult neurogenesis in mammals is a highly conserved activity, existing from lower rodents\[^{42-43}\] to primates\[^{44-46}\]. Therefore, using specific markers such as Pax6 (a transcription factor during embryonic development)\[^{47-48}\] and TuJ 1 (the neuron-specific class III-tubulin)\[^{49-50}\] to visualize the different stages of neurons in adult neurogenesis is a useful tool to understand the details and mechanisms of adult neurogenesis. As a microtubule-associated protein\[^{51}\], doublecortin can be used to differentially detect the proliferative progenitor cell stage from the post-mitotic phase with long dendrites\[^{52-53}\]. That is to say, doublecortin immunoreactivity can be found both in proliferating progenitor cells and in immature newborn neurons. Furthermore, based on the location and apical dendrites\[^{54-55}\], doublecortin-positive cells can be divided into two types in a simple way. Smaller cells (class I cells) without a dendrite that penetrate the granule cell layer are known to be progenitor cells, since 2/3 of the population of these types of cells are in the cell cycle. A later study\[^{54}\] also showed younger neurons expressed lesser levels of doublecortin using immunofluorescence cell sorting. Therefore, 2/3 of class I cells labeled by doublecortin could be regarded as proliferating progenitor cells. Longer cells (class II cells) with dendrites extending into the molecular layer are considered as immature post-mitotic neurons (Figure 1B). Here, our data found that in corticosterone-treated rats, the number of class II cells was greatly decreased while leaving the class I cells nearly untouched. The data indicated that corticosterone injection impaired neurogenesis in the hippocampus, mainly by reducing the number of newly born neurons. However, after baicalin treatment, both the number of class I and class II doublecortin-positive cells were dramatically increased. The findings strongly suggest that baicalin might promote the survival of both the proliferating progenitor cells and post-mitotic neurons in adult neurogenesis, and consequently increase the number of both class I and class II doublecortin-positive cells in the hippocampus. A few previous studies found that baicalin treatment was able to promote the survival of adult neurons. Jung \textit{et al.}\[^{55}\] performed a study showing that baicalin protected retinal ganglion cells \textit{in vitro} from ischemic and oxidative insults. Other studies also found that baicalin treatment enhanced the survival of cortical neurons.
under deprivation of oxygen/glucose or glutamate toxicity\textsuperscript{[56-57]}. Here, our data added to the knowledge that baicalin could enhance the survival of proliferating progenitor cells and newly formed neurons during the adult neurogenesis in the hippocampus. In addition, the dendritic toxicity of corticosterone has long been recognized in mature neurons in the adult central nervous system\textsuperscript{[58-59]}. Previous studies have shown that daily injection or oral administration of corticosterone greatly impairs apical dendritic morphology of the pyramidal cells in the CA3 region of the hippocampus. Corticosterone treatment decreased the length and branches of apical dendrites in CA3 pyramidal cells\textsuperscript{[60-61]}. Similar changes were also found in CA1 pyramidal cells in the adult hippocampus\textsuperscript{[62-63]}. The reduced dendritic length and branches in mature pyramidal cells caused by corticosterone were further thought to be related to the release of excitatory amino acids\textsuperscript{[64]}. More recently, studies have also reported that corticosterone was able to affect spine stability and dendritic morphology in developing neurons\textsuperscript{[65-67]}. In spite of mature neurons, research has proven that corticosterone treatment is also able to affect the dendritic dynamics in developing neurons. In young hippocampal organic cultures, high-dose application of corticosterone induced the atrophy of the apical dendritic tree in CA1 pyramidal cells in tissue slices\textsuperscript{[66]}. Another example of corticosterone inhibiting dendritic development in young neurons was in prenatally stressed experimental animals\textsuperscript{[68-71]}. Offspring animals with prenatal stress were shown to display elevated serum corticosterone and were found to have reduced spine density and dendritic complexity in the hippocampus\textsuperscript{[70]}, as well as in the prefrontal cortex\textsuperscript{[71]}. In the present study, our data also found that the reduced dendritic complexity in doublecortin-positive neurons was induced by 2-week corticosterone injection. Considering the criteria in analyzing the doublecortin-positive cells with tertiary dendrites, the qualified doublecortin-positive neurons were more likely to be class II doublecortin-positive cells, which were newly formed neurons during the process of neurogenesis. In the current study, after baicalin treatment, the dendritic damage to class II doublecortin-positive cells caused by corticosterone injection was reversed. This finding indicated that corticosterone injection impaired the dendritic development of the newly formed neurons during the process of neurogenesis; while baicalin treatment protected the dendritic morphology of the doublecortin-positive cells in adult neurogenesis from corticosterone toxicity. Previous studies have shown that by using antagonists of corticosterone receptors (RU38468) or N-methyl-D-aspartic acid receptor knockdown mice, the inhibitory effects of corticosterone on dendritic growth were blocked in the CA1 and CA3 fields, respectively\textsuperscript{[66, 72]}. Additionally, it was well established by Garcia and colleagues that the glucocorticoid receptors were abundantly expressed in the newly formed neurons generated from the neural progenitor cells during neurogenesis\textsuperscript{[73]}. An in vitro study has shown that, similar to MK801 (an antagonist of N-methyl-D-aspartic acid receptor), baicalin exhibited the ability to inhibit the activation of 5-lipoxygenase mediated by the N-methyl-D-aspartic acid receptor, and consequently reduced the damage by oxygen/glucose deprivation on the cultured cortical neurons\textsuperscript{[74]}. Hence, one possible explanation here is that baicalin might antagonize the adverse effects of corticosterone on the dendritic development of young neurons by modulating the bioactivity of the N-methyl-D-aspartic acid receptors or the glucocorticoid receptors, or both. Altogether, the present in vivo study showed that baicalin exhibited beneficial effects on proliferating progenitor cells and newborn neurons during adult neurogenesis in the dentate gyrus. Whether baicalin has the potential to treat psychological disorders or to improve cognitive functions requires further investigation, such as behavioral tests and cell signaling studies.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
All the experiments were conducted from September 2011 to May 2012 in the Anesthesia Research Institute, Central South University, China.

**Materials**

**Animals**
Twelve 2-month-old male Sprague-Dawley rats, weighing 220–250 g, were obtained from the Department of Laboratory Animal at Central South University, China, with license number SCXK (Xiang) 2009-0004. The animals were maintained at a controlled temperature and 12-hour light/dark cycle with access to food and water ad libitum. All experimental procedures were in accordance with the Guidance Suggestions for the Care and Use of...
Laboratory Animals, formulated by the Ministry of Science and Technology of China[75].

**Drug**

Baicalin, 95% purity, was purchased from Sigma-Aldrich (Cat No. 572667, St. Louis, MO, USA).

**Methods**

**Establishment of corticosterone-induced stress models and drug treatment**

Corticosterone (Sigma-Aldrich) was suspended and sonicated in sesame oil as previously described[13]. The ready-to-use suspension was subcutaneously injected into PBS + corticosterone and baicalin + corticosterone groups at a dose of 40 mg/kg daily for 14 days to induce stress. At the same time, an oral feeding with 50 mg/kg baicalin dissolved in 0.01 M PBS was started, and was given to the baicalin + corticosterone group for 14 days. The PBS + corticosterone rats were orally given 0.01 M PBS at the same volume. The PBS + vehicle rats were orally given 0.01 M PBS plus vehicle injection (sesame oil) for 14 days; the baicalin + vehicle rats were orally dosed with 50 mg/kg baicalin dissolved in 0.01 M PBS plus vehicle injection for 14 days.

**Sample collection and tissue processing**

After treatment, all animals were anesthetized by over-dose of 2% sodium phenobarbital and transcardially prefixed with 4% paraformaldehyde. All the brains were post-fixed for 48 hours in 4% paraformaldehyde and immersed in 30% sucrose solution until each brain sank to the bottom of the solution. Serial coronary frozen sections (40 µm thickness) were cut with a freezing microtome and prepared for immunohistochemistry.

**Immunohistochemistry for doublecortin expression**

The brain sections were brought to room temperature and were blocked with a solution containing 0.01 M PBS, 0.1% Triton X-100 and 10% normal goat serum solution for 30 minutes, and subsequently incubated at 4°C overnight with polyclonal rabbit anti-doublecortin (1:500; Cell Signaling Technology, Boston, MA, USA). After thorough washes, sections were then incubated with goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) and followed by avidin-biotin complex (1:200; Vector Laboratories) at room temperature for 60 minutes, respectively. Chromogen reactions were performed with diaminobenzidine (Sigma-Aldrich) and 0.1% H₂O₂ for 10 minutes. The sections were dehydrated in graded alcohol and cleared in toluene, and then coverslipped. The stained sections were observed using a CHBS light microscope (Olympus, New York Microscope Company Inc., NY, USA).

**Analysis of the number of doublecortin-positive cells**

To determine the number of doublecortin-positive cells in the hippocampus, six sections (at 440 µm intervals) were selected in each animal and processed for immunohistochemical staining. The doublecortin-positive cells in the dentate gyrus in the dorsal part of hippocampus were counted using Stereo Investigator software (Micro-Bright Field Biotechnology, New York, NY, USA) by a skilled and blinded experimenter. The software randomly offered 30–50 visual fields under 40 × lens and the qualified cells were counted. The selected doublecortin-positive cells were further divided into class I and class II doublecortin-positive cells, as previously described[35, 53]. Briefly, the most mature class II doublecortin-positive cells displayed a primary dendrite that was orientated perpendicular to the subgranular zone. The class I doublecortin-positive cells were located in the subgranular zone, without a dendrite, or only a short dendrite.

**Dendritic complexity of doublecortin-positive neurons**

Dendritic outline of doublecortin-positive cells including dendritic length and number of intersections (branch points) in the granule cells layer of the hippocampal dentate gyrus were traced using Filament tracer software (Bitplane Inc., South Windsor, CT, USA) and then analyzed by Imaris track software (Bitplane Inc.). A qualified neuron for analysis displayed a comparatively independent dendritic tree with at least tertiary branches. Tracings were analyzed by Sholl analysis in concentric circles (diameter 10–200 µm) with their center in the cell body[39]. In brief, the dendritic length counted as the length of the tracing between every two concentric circles. The intersection number was the number of tracings crossing every circle. All tracings were done by a skilled experimenter blind to the groups and treatments. A higher value in dendritic length or the number of intersections represented a neuron with more complex dendritic branches.

**Statistical analysis**

All data were presented as mean ± SEM. Comparisons of multiple groups were done by two-way analysis of variance-dependent experimental designs and followed by Student-Newman-Keuls test for intergroup comparisons with SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). Significance was set at a probability level of P < 0.05.
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**Author contributions:** Yaping Wang and Xinghua Jiang designed the experiment. Yaping Wang and Junmei Xu helped to revise the manuscript. Dingquan Zou and Lin Yang helped Xinghua Jiang to conduct the experiment and participated in the cell tracings. Xinghua Jiang analyzed the data and wrote the manuscript. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** All protocols were approved by the Animal Ethics Committee of Central South University in China.

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