Leonurine promotes neurite outgrowth and neurotrophic activity by modulating the GR/SGK1 signaling pathway in cultured PC12 cells

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Depression is a common psychiatric disorder that affects almost 10% of children and adolescents worldwide. Numerous synthetic chemical antidepressants used to treat depression have adverse side effects. Therefore, new therapeutic approaches for depression treatment are urgently needed. Leonurus cardiaca has recently been shown to be effective for the treatment of nervous system diseases such as depression, but its mechanism is not clear. In this study, we aimed to reveal the mechanism underlying leonurine's antidepressant activity. Leonurine was used to treat corticosterone-induced PC12 cells to examine its effect on neurite outgrowth and neurotrophic factors after treatment with the inhibitor of glucocorticoid receptor (GR) and serum-inducible and glucocorticoid-inducible kinase 1 (SGK1). Methyl thiazolyl tetrazolium assays were used to evaluate the viability of cells. High content analysis was used to detect cell area, total neurite length, maximum neurite length, and expression of GR, SGK1, brain-derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3), and B-cell lymphoma-2 (BCL-2). The results showed that leonurine increased cell viability in a concentration-dependent manner, with the maximal prosurvival effect at 60 µM. Leonurine increased cell area, total neurite length, and maximum neurite length of corticosterone-induced PC12 cells, increased the expression of GR, BDNF, NT-3, and BCL-2, and decreased the expression of SGK1. After treatment with GR inhibitor RU486, the expressions of GR, BDNF, NT-3, and BCL-2 were significantly decreased and SGK1 was increased. In contrast, treatment with GSK650394 had the opposite effect of RU486. Our data indicate that leonurine promotes neurite outgrowth and neurotrophic activity in cultured PC12 cells, and its potential mechanism may involve the GR/SGK1 signaling pathway. NeuroReport 30:247–254 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Depression is a common and major psychiatric disorder characterized by symptoms involving sleep disturbance, loss of appetite, lack of interest, low self-worth, and even suicidal thoughts [1,2]. The global prevalence of depression is 4.7% and the pooled annual incidence is 3.0% [3], indicating that ~350 million individuals are impacted by depression worldwide [4]. Stress is a major trigger of depression, which manifests itself as the body's response to a stimulus shown in the form of a mental and/or an emotional response [5].

The hypothalamic–pituitary–adrenal (HPA) axis is a neuroendocrine system involved in the production of the stress hormone cortisol. The steady state of the HPA axis is regulated by the glucocorticoid receptor (GR) through negative feedback in the hippocampus. However, stress-induced progressive HPA axis abnormalities can lead to high circulating levels of cortisol and high expression of GR, which not only triggers depression and anxiety-like changes in behavior [6] but also causes changes in the brain structure because of decreased neurotrophic activity. Serum-inducible and glucocorticoid-inducible kinase 1 (SGK1) is not only stimulated by glucocorticoids and serum but is also a downstream target of GR. SGK1 participates in the occurrence of depression through the glucocorticoid signaling pathway and maintains GR activity potentially without glucocorticoids by regulating GR phosphorylation levels [7]. The GR/SGK1 signaling pathway is implicated in learning, memory, and neuroplasticity as well as the stress response and depression.

Neurite outgrowth has received the most attention as an indicator of neurodevelopment and neuroregeneration *in vitro* as the development of axonal and dendritic processes is a defining characteristic of neuronal cell morphology and a critical determinant of neuronal cell connectivity and function [8]. The GR antagonist RU486 was shown to counteract the inhibitory effect of dexamethasone pretreatment on neurite extension from PC12 cells [9]. Neurotrophic factors are vital for supporting neuronal survival and play a role in the process of regulating neuronal formation in neural networks. SGK1 acts downstream from corticosterone (CORT) to induce morphological changes in nerve cells [10]. SGK1 regulates the neurotrophic support of hippocampal neurons by regulating brain-derived neurotrophic factor (BDNF) [11]. In addition, the hippocampal shrinkage observed commonly in patients with depression has been linked to decreased neurotrophic support in association with high levels of cortisol [12,13]. Also, clinic antidepressants fluoxetine has been shown to promote neurite outgrowth and regulate expression of the neurotrophic factors [14].

Leonurus cardiaca, a herbaceous perennial plant in the mint family, has a long history of use in traditional medicine in the treatment of a variety of diseases in China, Japan, Korea, and European countries. Leonurine, also called SCM-198 (4-guanidino-*n*-butyl syringate), is a chemically synthesized compound based on a bioactive alkaloid extracted from *L. cardiaca* [15]. Leonurine has recently been shown to be effective in the treatment of cardiovascular and nervous system disease [16]. Jia *et al.* [17] found that leonurine exerts antidepressant effects in a stress-induced depression animal model. This study also found that the ability of leonurine to ameliorate behavioral parameters was related to learning and memory, and that its mechanism mainly involved increasing monoamine neurotransmitters and inhibiting neuroinflammation. However, very few studies have examined the underlying molecular mechanisms. Therefore, in the present study, we hypothesized that leonurine could attenuate the negative effects of CORT on neurite outgrowth and neurotrophic molecules in PC12 cells by modulating the expression of GR and SGK1.

Materials and methods
Chemicals and reagents
Leonurine and CORT with purities of 98% were purchased from Sigma-Aldrich (St Louis, Missouri, USA). GR inhibitor RU486 and SGK1 inhibitor GSK650394 were also purchased from Sigma-Aldrich. Fluoxetine hydrochloride was acquired from Tianjin Tasly Pharmaceutical Co., Ltd (Tianjin, China). Leonurine was dissolved in DMSO (Sigma-Aldrich) and ethanol (50% v/v) and diluted in saline at a concentration of 20 μmol/ml.

Cell culture and drug treatments
PC12 cells originating from rat adrenal medulla were obtained from Procell Life Science & Technology Co., Ltd.

(Wuhan, China). All cell culture reagents were obtained from Life Technologies (Grand Island, Nebraska, USA). Cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 5% fetal bovine serum (Gibco, Grand Island, Nebraska, USA) and 10% horse serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified CO_2 (5%) incubator at 37°C.

PC12 cells are typically used to establish a depression model *in vitro*, which is accomplished by the administration of CORT [18]. PC12 cells were treated with 300 μM CORT for 24 h. Cell viability decreased to ~50% at this concentration of CORT, which was therefore used in all subsequent experiments *in vitro*.

Part 1: To determine the effect of leonurine in a major depression model, the cells were divided into five groups: normal control, CORT + PBS, CORT + fluoxetine, and CORT + leonurine (10, 20, 40, 60, 80, and 100 μM). Analysis was carried out 24 h after the cells were seeded. Leonurine was applied 2 h before CORT treatment and the cells were cultured for another 24 h. Viability was then determined using an MTT assay.

Part 2: To research the molecular mechanism of leonurine in a major depression model, the cells were divided into seven groups: nontreated nornal (Normal), CORT + PBS (PBS), fluoxetine serum group (FLU), leonurine group (LEO), leonurine plus GR inhibitor group (LEO + RU), and leonurine + SGK inhibitor group (LEO + GSK). Drugs were diluted with neurobasal medium, and the culture medium was replaced with fresh neurobasal medium 3 h before any drug treatment.

Tetrazolium (MTT)-based colorimetric cell viability assay
Cell viability was assessed using an MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide] assay. Cells were seeded in 96-well plates for 24 h and treated with drugs for 72 h before adding MTT. The cells were then incubated with MTT for another 3 h at 37°C. Thereafter, absorbance at 570 nm was measured in a microplate reader (Thermo Scientific, Fremont, California, USA).

Cell morphology, neurite outgrowth, and expression of immunofluorescent proteins
A set of tests are commercially available to evaluate cell morphology, neurite outgrowth, and expression of immunofluorescent proteins using a high content analysis (HCA) system (PerkinElmer, Boston, Massachusetts, USA). The method utilizes automated measurements in 96-well plates stained using the immunocytochemical procedure described below.

Following drug exposure, cells in transparent plates were fixed for 20 min in a fixative solution consisting of 4% paraformaldehyde and 10 μg/ml Hoechst 33342 in 1× PBS. Cell bodies were labeled using an anti-β-tubulin primary antibody (1 : 150; Proteintech, Chicago, Illinois, USA) and antibodies against GR, SGK1, BDNF,

neurotrophic factor-3 (NT-3), and B-cell lymphoma-2 (BCL-2) (all 1 : 500; Abcam, Cambridge, UK), followed by an FITC-conjugated secondary antibody protected from light. The plate was washed three times with 100 μl of 1 × PBS after each operation, retaining the buffer from the final wash. Plates were then loaded into the Operetta HCA device (PerkinElmer, Boston, Massachusetts, USA) for image capture and analysis.

The assay parameters cited in this method are associated with an optimized image analysis protocol for measuring neurite outgrowth in differentiated NS-1 cells. The acquired images were analyzed using the Harmony system (PerkinElmer, Boston, Massachusetts, USA) of HCA to measure the cell area, total neurite length, and maximum neurite length of all identified cell in a plate. Fluorescence images were produced using a multiple bandpass mission filter and matched excitation filters for nuclei and cell bodies, and then acquired using a high-resolution charge-coupled device camera. The system uses an automated inverted epifluorescence microscope to focus and record images from multiple fields in each individual well.

Data analysis
Data are presented as mean ± SEM. Statistical analysis of the data was carried out by one-way analysis of variance, followed by Fisher's least significant difference test our in test. all analyses were carried out using SPSS 20.0 (IBM, Armonk, New York, USA). A *P* value less than 0.05 was considered a statistically significant difference.

Results
Leonurine reversed CORT-induced cell death in PC12 cells
PC12 cells are used commonly for the establishment of depression models *in vitro* when they are combined with the administration of CORT [19]. To obtain an appropriate depression model, PC12 cells were treated with different concentrations of CORT. When treated with 400 μM CORT for 24 h, cell viability decreased to ~ 50% (Fig. 1a); thus, this concentration was used in subsequent experiments *in vitro*.

The MTT assay was performed to investigate the damage to PC12 cells at different concentrations of CORT and the effects of leonurine on CORT-induced cell death. When PC12 cells were exposed to CORT at 400 μM for 24 h, cell viabilities of the different groups (Normal, PBS, LEO 10, 20, 40, 60, 80, and 100 μM) were 100, 49.1, 52.3, 56.8, 64.4, 75.4, 63.2, and 60.7%, respectively. The cell viability of the PBS group was significantly lower than the others, whereas leonurine increased cell viability in a concentration-dependent manner. The prosurvival effect of leonurine was observed at 60 μM (Fig. 1b).

Leonurine promoted CORT-induced cell neurite outgrowth in PC12 cells
Neurite outgrowth is a critical cellular process underlying nervous system development that can be quantified by HCA using automated microscopy and image analysis [20]. Images were captured automatically using the Array Scan HCA platform (PerkinElmer, Boston, Massachusetts, USA) and analyzed using the Harmony system, which contains morphological endpoints including cell area, total neurite length, and maximum neurite length.

The outgrowth of axonal and dendritic processes (neurites) is a hallmark of neuronal differentiation and maturation [21]. CORT induced a significant decrease in total neurite length [$F(2,9) = 110.1$, $P = 0.001$], maximum neurite length [$F(2,9) = 180.2$, $P = 0.001$], and cell area [$F(2,9) = 164.3$, $P = 0.001$] compared with the normal group. The effects of leonurine on neurite outgrowth *in vitro* are shown in Fig. 2. Leonurine promoted total neurite outgrowth [$F(3,12) = 87.7$, $P = 0.007$] and maximum neurite length [$F(3,12) = 146.6$, $P = 0.001$], and also increased cell area [$F(3,12) = 151.4$, $P = 0.002$] compared with the PBS group. This action was strengthened after treatment with the SGK1 inhibitor GSK650394, LEO + GSK650394 promoted total neurite

Fig. 1
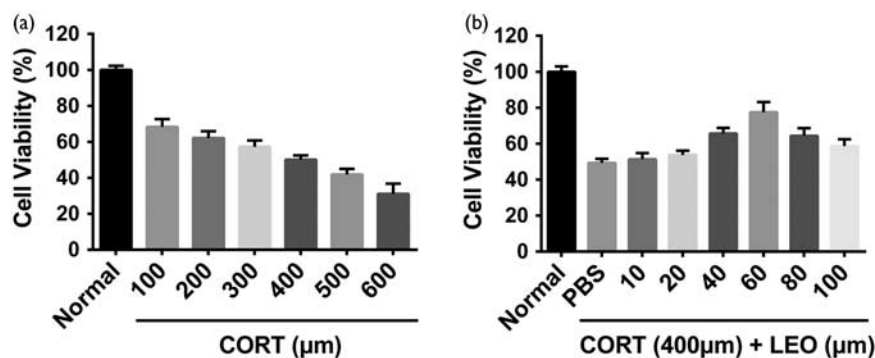
The effect of corticosterone (CORT) on PC12 cell viability (a) and the effect of leonurine (LEO) on CORT-induced changes in PC12 cell viability (b), as determined using an MTT assay. The results are expressed as mean ± SEM.

Fig. 2
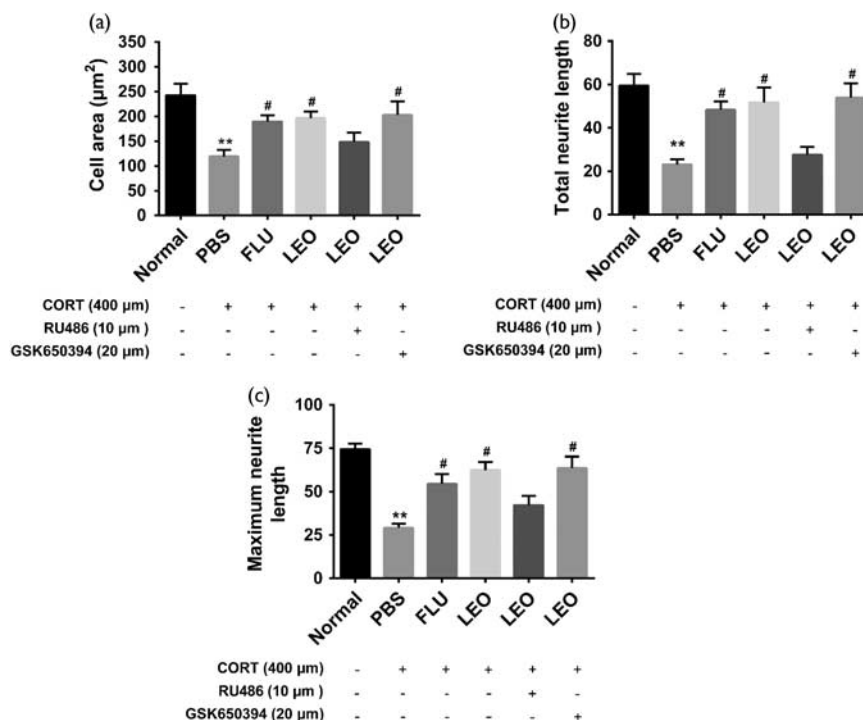
Protective effect of leonurine on corticosterone (CORT)-induced changes in PC12 cell morphological endpoints. Cell area of each group (a); total neurite length of each group (b); and maximum neurite length (c). The results are expressed as mean ± SEM. **$P < 0.01$, compared with the normal group; #$P < 0.05$, compared with the PBS group. FLU, fluoxetine serum group; LEO, leonurine group.

outgrowth [$F(4,15) = 76.6$, $P = 0.007$] and maximum neurite length [$F(4,15) = 132.7$, $P = 0.004$], and also induced an increase >in the cell area [$F(4,15) = 257.5$, $P = 0.001$] compared with the PBS group. However, the effect of LEO + GSK650394 on total neurite outgrowth [$F(4,15) = 76.6$, $P = 0.34$], maximum neurite length [$F(4,15) = 132.7$, $P = 0.69$], and cell area [$F(4,15) = 257.5$, $P = 0.16$] showed no significant difference compared with the LEO group (Fig. 2).

In contrast, after treatment with GR inhibitor RU486, leonurine exerted the opposite effect on morphological endpoints compared with GSK650394. LEO + RU486 inhibited total neurite outgrowth [$F(4,15) = 106.7$, $P = 0.002$] and maximum neurite length [$F(4,15) = 107.1$, $P = 0.001$], and also decreased cell area [$F(4,15) = 289.2$, $P = 0.001$] compared with the PBS group. However, the effect of LEO + RU486 on total neurite outgrowth [$F(4,15) = 106.7$, $P = 0.15$], maximum neurite length [$F(4,15) = 107.1$, $P = 0.48$], and cell area [$F(4,15) = 289.2$, $P = 0.32$] showed no significant difference compared with the LEO group (Fig. 2).

The protective effect of leonurine was influenced by inhibiting GR and SGK1

First, we evaluated whether GR and SGK1 were involved in the effect of leonurine on PC12 cells after different drug treatments. We pretreated the cells with inhibitors of GR (RU486, 10 μM) and SGK1 (GSK650394, 20 μM), and then exposed them to CORT (400 μM) in the presence or absence of leonurine (60 μM) for 40 min. Cell viability was determined using an MTT assay. PBS + CORT induced a significant decrease in cell viability (49.1% compared with the normal group), whereas leonurine increased cell viability (73.4% compared with the normal group). Notably, the protective effect of leonurine against CORT-induced cell death was attenuated by RU486 (52.2% compared with the normal group), but this effect was strengthened by GSK650394 (76.5% compared with the normal group), suggesting the involvement of both GR and SGK1 in the effect of leonurine (Fig. 3a). The cell viability was decreased in the PBS [$F(2,9) = 408.7$, $P = 0.002$] group, whereas LEO [$F(3,12) = 355.4$, $P = 0.004$] and LEO + GSK650394 [$F(3,12) = 355.4$, $P = 0.005$] induced a significant increase in cell viability compared with the PBS group.

We then examined whether leonurine could protect PC12 cells by regulating neurotrophic proteins and nerve apoptosis through the GR/SGK1 signaling pathway. In our study, the expression of GR [$F(2,9) = 206.1$, $P = 0.002$], BDNF [$F(2,9) = 77.8$, $P = 0.003$], NT-3 [$F(2,9) = 110.3$, $P = 0.001$], and BCL-2 [$F(2,9) = 353.1$, $P = 0.001$] in CORT-cultured PC12 cells was low in the PBS group, whereas the expression of SGK1 [$F(2,9) = 181.1$, $P = 0.001$] increased. After treatment with leonurine, the expression of GR [$F(2,9) = 206.1$, $P = 0.001$], BDNF [$F(2,9) = 77.8$, $P = 0.001$],

Fig. 3
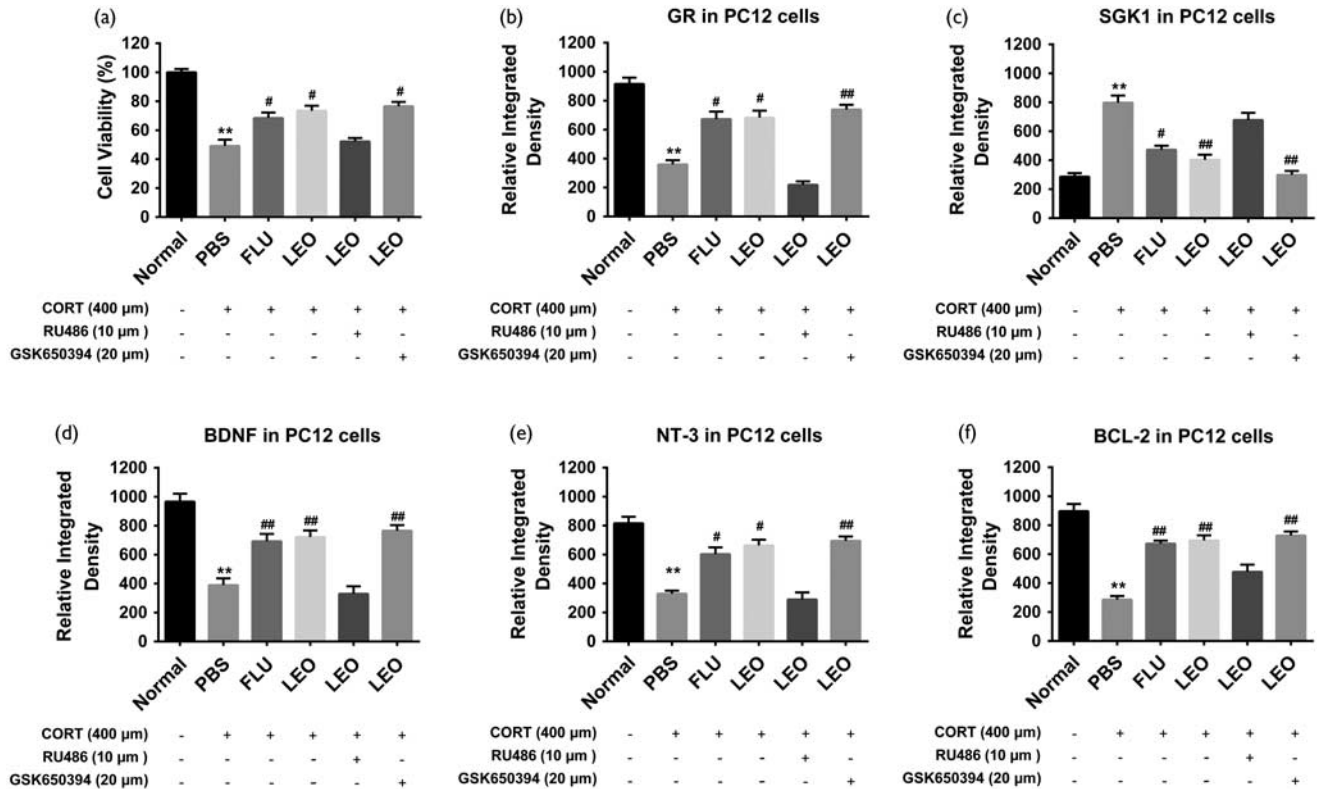
The effect of leonurine on corticosterone (CORT)-induced changes in PC12 cell viability determined using an MTT assay after treatment with RU486 and GSK650394 (a). Relative integrated density of GR (b), SGK1 (c), BDNF (d), NT-3 (e), and BCL-2 (f) in PC12 cells after treatment with RU486 and GSK650394. The results are expressed as mean ± SEM. ** $P < 0.01$, compared with the normal group; # $P < 0.05$, ## $P < 0.01$, compared with the PBS group. BCL-2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; FLU, fluoxetine serum group; GR, glucocorticoid receptor; LEO, leonurine group; NT-3, neurotrophic factor-3; SGK1, serum-inducible and glucocorticoid-inducible kinase 1.

NT-3 [$F(2,9) = 110.3$, $P = 0.001$], and BCL-2 [$F(2,9) = 353.1$, $P = 0.002$] increased and that of SGK1 [$F(2,9) = 181.1$, $P = 0.004$] decreased (Figs 3 and 4).

However, after treatment with RU486, LEO induced a significant decrease in the expression of GR [$F(4,15) = 88.3$, $P = 0.003$], BDNF [$F(4,15) = 134.7$, $P = 0.001$], NT-3 [$F(4,15) = 146.8$, $P = 0.002$], and BCL-2 [$F(4,15) = 297.5$, $P = 0.005$] and induced an increase in the expression of SGK1 [$F(4,15) = 157.9$, $P = 0.006$], but there were no differences between LEO treatment with RU486 or not for all five indexes ($P > 0.05$).

Compared with treatment with RU486, GSK650394 had the opposite effect (Figs 3 and 4). LEO + GSK650394 promoted the expression of GR [$F(4,15) = 194.3$, $P = 0.003$], BDNF [$F(4,15) = 79.6$, $P = 0.004$], NT-3 [$F(4,15) = 138.5$, $P = 0.002$], and BCL-2 [$F(4,15) = 280.9$, $P = 0.001$], and decreased the expression of SGK1 [$F(4,15) = 148.7$, $P = 0.003$] compared with the PBS group. However, the effect of LEO + GSK650394 on GR [$F(4,15) = 194.3$, $P = 0.007$], BDNF [$F(4,15) = 79.6$, $P = 0.007$], NT-3 [$F(4,15) = 138.5$, $P = 0.007$], BCL-2 [$F(4,15) = 280.9$, $P = 0.007$], and SGK1 [$F(4,15) = 148.7$, $P = 0.007$] showed no significant difference compared with the LEO group (Fig. 2).

Discussion

The present results show that CORT caused neurotoxicity in PC12 cells, whereas leonurine prevented cell death mediated by GR/SGK1 signaling. This conclusion was supported by the following observations: (i) treatment with CORT in PC12 cells caused cell death, whereas leonurine significantly reversed the toxic effect of CORT; (ii) inhibition of GR blocked the neuroprotective effect of leonurine on CORT-induced PC12 cell death, whereas inhibition of SGK1 promoted the effect of leonurine; (iii) leonurine exerted a significant protective effect on PC12 cells, increasing the levels of BDNF, NT-3, and BCL-2, while decreasing the expression of SGK1. All of these effects of leonurine were essentially identical to those observed with fluoxetine. The present study confirmed that CORT causes neurotoxicity in PC12 cells accompanied by damaged cell morphology, reduced neurite outgrowth, and disruption of the GR/SGK1 signaling pathway.

CORT is associated closely with the occurrence of depression [22]. Administration of CORT has been used to establish

Fig. 4
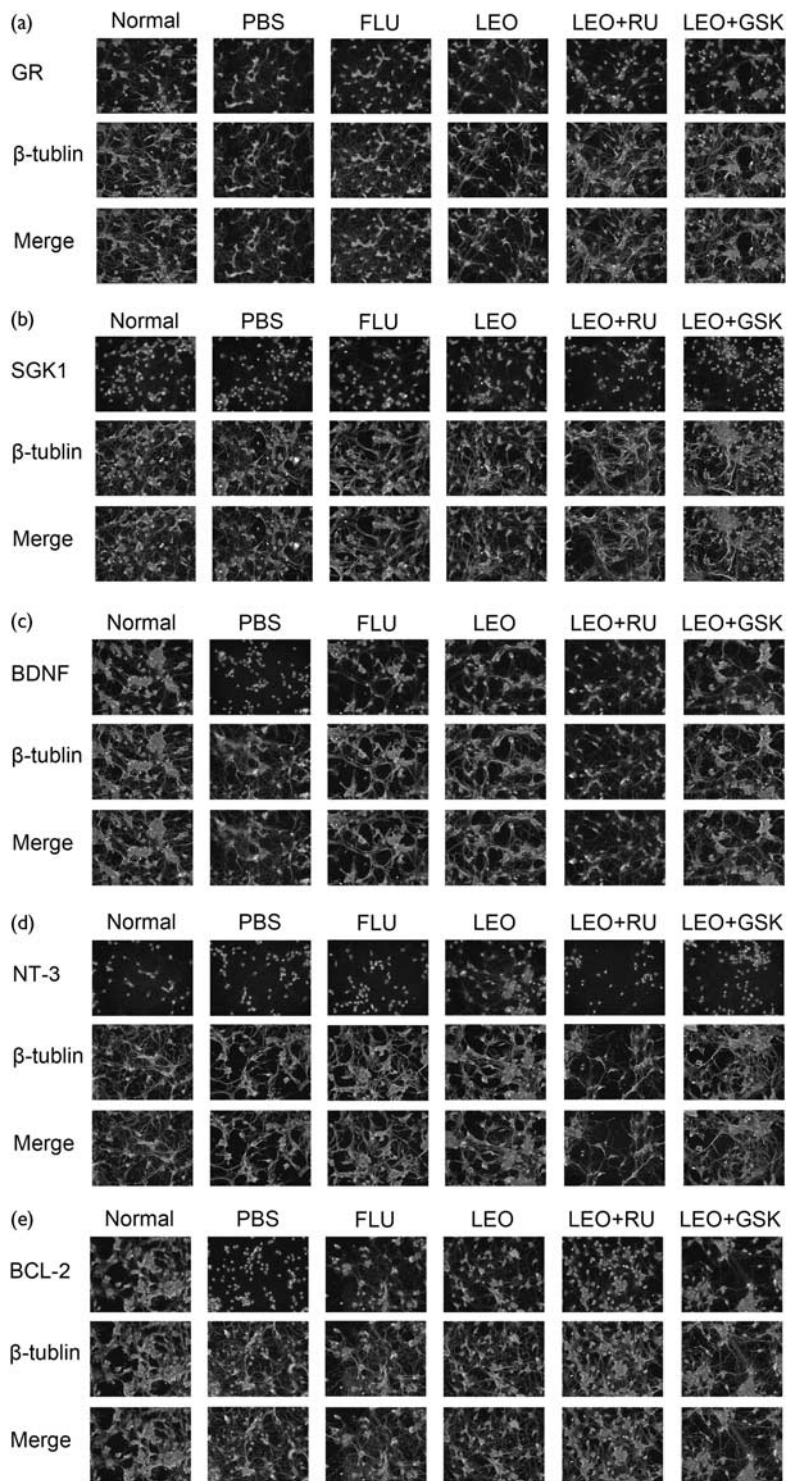
(a-e) Representative immunofluorescence images for GR, SGK1, BDNF, NT-3, and BCL-2 expression in each group after treatment with RU486 and GSK650394. BCL-2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; FLU, fluoxetine serum group; GR, glucocorticoid receptor; LEO, leonurine group; LEO + GSK, leonurine + SGK inhibitor group; LEO + RU, leonurine + GR inhibitor group; NT-3, neurotrophic factor-3; SGK1, serum-inducible and glucocorticoid-inducible kinase 1.

models of major depression *in vitro* and *in vivo* [23,24]. The PC12 cell line is widely used as a model system to study a variety of neuronal functions, and given the high level of GRs expressed in PC12 cells, they are very sensitive to glucocorticoid exposure [25,26]. It has been reported that CORT can induce apoptosis and damage in PC12 cells and produce depression-like behavior in animal models [27,28]. Drugs that can reverse CORT-induced neurotoxicity may thus have therapeutic potential for preventing or treating depression.

Considerable data suggest that excessive and prolonged chronic stress results in hyperactivity of the HPA axis, which may be involved in the pathogenesis of depression [29,30]. Cortisol exerts direct toxic effects on the brain, such as reduced neurotropic factors and neuroplasticity, and also promotes apoptosis [31]. Indeed, the average concentration of cortisol is reportedly higher in depressed patients than in healthy controls [32]. On the basis of the critical role of GR in the HPA axis and in mediating the effects of glucocorticoids on the brain, it is noteworthy that GR is a potential target for antidepressant drugs [33]. SGK1 is a mediator of the effects of glucocorticoids on GR function and neurogenesis, and it also acts as a key intermediary between stress and depression [34]. Accumulating studies have shown that SGK1 may be a downstream regulator of glucocorticoids and may play a role in the partial effects of glucocorticoids on brain function [35,36].

Hippocampal injury is closely related to depression, which is manifested by hippocampal nerve regeneration disorder and neurotrophic and synaptic plasticity deficits. Interestingly, SGK1 has been reported to be correlated negatively with BDNF, which may provide a potential mechanism for the impaired neurogenesis observed in depression [37]. BDNF and NT-3 are members of the neurotrophin family that serve as biomarker proteins closely related to depression. A large number of preclinical studies have shown that a variety of stressors can reduce the activity of the BDNF and NT-3 pathway in the hippocampus, and that antidepressants can enhance pathway activity [38,39]. Neurotrophic and neural apoptotic activity can thus interact with each other. BDNF can affect neural apoptosis by regulating the expression of BCL-2 [40].

Leonurine exerts neuroprotective effects on ischemic stroke, Parkinson's disease, and Alzheimer's disease in animal models [41,42], and can rescue behavioral deficits in animals, promote neuronal survival, and modulate inflammation. However, the effect of leonurine on neuropsychiatric disorders, particularly major depression, remains unknown. Therefore, we used CORT-cultured PC12 cells exposed to GR and an SGK1 inhibitor to study the antidepressant mechanisms of leonurine. Our results showed that leonurine impacted the expression of GR, BDNF, NT-3, BCL-2, and SGK1, whereas RU486 and GSK650394 inhibited and promoted this activity, respectively. Taken together, our results suggest that leonurine exerts antidepressant effects that may control the GR/SGK1 signaling pathway to regulate neurite outgrowth and neurotrophic activity in CORT-cultured PC12 cells.

Conclusion

The present study indicated that leonurine showed antidepressant-like properties in a CORT-induced depression model in PC12 cells, which produced beneficial effects on neurite outgrowth and neurotrophic factors. The action of leonurine was possibly mediated by its ability to increase GR, BDNF, NT-3, and BCL-2 levels while decreasing the expression of SGK1. The underlying mechanism may involve the GR/SGK1 signaling pathway.

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Authors' contributions: Conceived and designed the experiments: Pan Meng, Xiaodan Liu, and Jingying Fan. Performed experiments: Pan Meng, Qing Zhu, Dan Liu, and Hui Yang. Analysis and interpretation of data: Pan Meng, Xiaoyuan Lin, and Wei Su. Writing and review of the manuscript: Pan Meng, Hui Yang, Xiaoyuan Lin, and Wei Su. Study supervision: Yuhong Wang, Pan Meng, and Qing Zhu.

Conflicts of interest

There are no conflicts of interest.

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