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Extractive from *Hypericum ascyron L* promotes serotonergic neuronal differentiation *in vitro*

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ABSTRACT

Background: Plant natural products have many different biological activities but the precise mechanisms underlying these activities remain largely unknown. *Hypericum longistylum* has long been recorded in Chinese medicine as a cure for depression and related disorders, but how it repairs neural lineages has not been addressed.

Methods: We extracted compounds from *Hypericum longistylum* and determined their effect on neural differentiation of embryonic stem cells (ESCs) *in vitro* by using the *Pax6-GFP* reporter system. The amount of serotonin released during differentiation was measured by HPLC. The tail suspension test and forced swimming test was performed for determining the effect of compounds on depression-like behaviors in mice.

Results: We found that one of the phloroglucinol derivatives not only facilitated differentiation of neural progenitor cells, but also increased the efficiency of differentiation into serotonergic neurons. This compound also improved the behaviors of mice placed in a stressful environment and reduced signs of depression.

Conclusions: This is the first use of Chinese herb derived-natural products to promote neurogenesis of ESCs, including the generation of serotonergic neurons, and the first attempt to identify the active compound in *Hypericum longistylum* responsible for its beneficial effects on depressive diseases.

1. Introduction

Since the discovery of artemisinin, Chinese medicine has attracted increasing global interest. Many natural products extracted from Chinese herbs show significant pharmacological activity and could potentially be used as medicines to cure diverse diseases (Xue and Roy, 2003; Cao et al., 2017). Depression is a type of mental illness that leads to severe and persistent low mood, which could lead to physical harm and increased risk of suicide (Steidtmann et al., 2012). The main cause of depression remains unclear and effective therapies are lacking. Three main types of drugs are currently used to treat depression: tricyclic antidepressants (Willner et al., 2013), monoamine oxidase inhibitors, and tetracyclic antidepressants. These drugs, however, only relieve the symptoms of depression and have severe side effects, including cardiovascular effects (Choi, 2003; Stahl, 2014). Chinese herbs are typically effective and have relatively few side effects, which is why scientists are increasingly trying to extract or synthetize the active components. Some Chinese herbs, such as *Hypericum longistylum*, have been shown to

Abbreviations: ESCs, embryonic stem cells; 5-HT, 5-hydroxytryptamine; MPLC, medium pressure liquid chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ECD, electronic circular dichroism; TDDFT, time-dependent density functional theory; EB, embryoid body; FACS, fluorescence-activated cell sorting; NPCs, neural precursor cells; RA, retinoic acid; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; NSC, neural stem cell; FGF4, fibroblast growth factor-4; SHH, sonic hedgehog; bFGF, basic fibroblast growth factor; FGF8, fibroblast growth factor-8; BDNF, brain-derived neurotrophic factor; SERT, serotonin transporter; TLC, thin layer chromatography; ODS, octadecylsilyl; LIF, leukemia inhibitory factor; TCA, trichloroacetic acid; MS, mass spectrometer

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have a positive effect on depression, but the active ingredient(s) and mechanism of action need to be further elaborated.

One possible cause of depression is the degeneration of serotonergic neurons, which would lead to a reduction in levels of 5-hydroxytryptamine (5-HT) in the central nervous system (Brambilla et al., 2010). Drugs that can increase levels of 5-HT have been shown to provide some benefits in depression (Li et al., 2006). *Hypericum longistylum* has many active chemical components, including phloroglucinol derivatives and, in the present study, we aimed to clarify whether these phloroglucinols can induce neural regeneration, especially of serotonergic neurons. Embryonic stem cells (ESCs) are pluripotent cells that can differentiate to neural lineages *in vitro* in the presence of growth factors (Li et al., 1998) and thus provide an ideal platform for the study of drug function and mechanisms. We therefore examined the effect of phloroglucinol derivatives, extracted from *Hypericum longistylum*, on serotonergic neuron differentiation in ESCs undergoing neural differentiation. We also tested the ability of one of these phloroglucinols to enhance the performance of mice in a stressed environment.

2. Material and methods

2.1. Plant materials

The aerial parts of *Hypericum longistylum* were collected from Changbaishan, Jilin Province, People's Republic of China, in August 2015. Botanical identification was carried out by one of the authors (X. Cao), and a voucher specimen (No. 20150803) was deposited at the laboratory of the Research Department of Natural Medicine, College of Pharmacy, Nankai University.

2.2. Mice

Specific pathogen-free (SPF)-grade mice were obtained from Beijing Vital River laboratory animal center and housed in the animal facilities of the Nankai University, China. Male and female mice of CD1 backgrounds at 8 week were used for tail suspension test and forced swimming test. The experiments were approved by the Animal Care and Use Committee of Nankai University, and all experiments conform to the relevant regulatory standards.

2.3. Extraction and isolation

The air-dried aerial parts of *Hypericum longistylum* (9.1 kg) were extracted with MeOH (3 × 80 L) under reflux. After evaporating the solvent, the residue (1000.0 g) was dissolved in H_2O (2.0 L) and extracted with petroleum ether (3 × 2.0 L). The petroleum ether soluble portion (309 g) was chromatographed on a silica gel column (silica gel, 100–200 mesh, 1.0 kg; column, 9 × 70 cm), using a petroleum ether-acetone step gradient system (100: 0, 100: 1, 100: 2, 100: 4, 100: 6, 100: 8, 100: 11, 100: 16, 100: 21, and 100: 26; 21 L for each gradient elution). Ten fractions (F1 − F10) were collected, guided by thin layer chromatography (TLC) analysis. Fraction F4 was subjected to MPLC, using an octadecylsilyl (ODS) column with a step gradient (68–95% MeOH in H_2O), to give seven sub fractions F4–1–F4–7. Sub fractions F4–2 were further purified by preparative HPLC (93% MeOH in H_2O) to provide compound 1 (t_R = 22 min; 21.4 mg yield). Fraction F6 was fractionated using the same MPLC conditions to give eight sub fractions, F6–1 − F6–8. Fraction F6–4 was further purified using the same HPLC system (90% MeOH in H_2O) to provide compound 2 (t_R = 31 min; 14.7 mg yield). The same MPLC and HPLC conditions were used to further separate fraction F5 into sub fractions F5–1 − F5–13. Further purification of F5–5 then yielded compounds 3 (t_R = 26 min, 39.4 mg) and 4 (t_R = 39 min, 14.0 mg).

2.4. Culture of mouse ESCs

Mouse ESCs (pax6-GFP) were grown on a mitotically inactivated mouse embryonic fibroblast feeder layer in an optimized stem cell growth medium (Ying et al., 2003). Briefly, the cells were grown in 1:1 mixture of DMEM/F12 (Gibco) supplemented with modified N2 (Gibco), and neurobasal medium (Gibco) supplemented with B27 (Gibco), supplemented with 5% knock out serum replacement (KOSR) (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich), 100 μM β-mercaptoethanol (Sigma-Aldrich), 100 mg/mL Strep-Pen (Gibco), 1500 U/mL leukemia inhibitory factor (LIF) (Millipore), 3 μM CHIR99021 (Gene-operation), and 1 μM PD0325901 (Gene-operation) at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. CCK-8 assay and DRAQ7 staining

A CCK-8 assay was used to measure the cytotoxicity of different concentrations of Compound 3 (Yang et al., 2014). Briefly, EBs that had been formed for 4 days were collected, resuspended in ES differentiation medium, and then re-plated on a fibronectin-coated dish. On the next day, when the EBs were attached on the bottom, the medium was aspirated and replaced with fresh medium (100 μL) containing different concentrations (2.5, 5, 10, 20, and 40 μM) of Compound 3. The cells were then incubated for an additional 72 h. Control wells contained medium alone, and three replicate wells were used at each concentration. After 72 h incubation, CCK-8 (10 μL, 5 mg/mL) was added to each well and the cells were incubated for another 3 h. The plate was then read using a BioTek luminescence reader (BioRed) at a test wavelength of 450 nm. For DRAQ staining, the cells were resuspended with culture medium at a concentration of no $> 5 \times 10^5$ cells/mL. DRAQ7 (CST) (1:100, 5 μL) was added to the cell suspension (0.5 mL), to a final concentration of 3 μM. The cells were gently mixed, incubated for 10 min on ice, and then analyzed directly in the presence of DRAQ7.

2.6. RNA isolation & real-time PCR

Total RNA from ESCs, ESC-derived neural precursors, and ESC-differentiated serotonergic neurons were isolated using Trizol reagent (Invitrogen). The cDNA was isolated using a Prime Script™ RT Reagent Kit with gDNA Eraser (Takara) for RT-PCR. RT-PCR was carried out using an ABI QuantStudio TM 6 Flex (ABI) with a FS Universal SYBR Green Master system (Roche). Relative expression levels were normalized to *Gapdh*. Average and SD are the result of three independent experiments. All primers are shown in Additional file 1: Table S1.

2.7. Immunocytochemistry

Cells were fixed overnight at 4 °C with 4% formaldehyde, washed three times with phosphate-buffered saline (PBS), and then incubated with blocking buffer (3% BSA) for 1 h at room temperature. The cells were then incubated overnight at 4 °C with rabbit anti-SERT antibody (Abcam), rinsed three times with PBS, and incubated with the corresponding secondary antibodies (Abcam) for 1 h at room temperature. After incubation, the samples were washed three times with PBS and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. The cells were examined under a fluorescent microscope and positive cells were counted by an investigator blinded to the identity of the samples. The data are the result of three independent experiments.

2.8. Neural differentiation of ESCs with a pax6-GFP reporter system

To generate NPCs, 6-/-ESCs (pax6-GFP reporter system), with well-defined morphology, were selected for differentiation. To form EBs, 6-/-ESCs were cultured with EB medium in non-coated petri dishes (Falcon) for 3 days. Briefly, the EB medium contained DMEM/F12 (Gibco),

supplemented with KOSR (20%), sodium pyruvate (1 mM; Sigma-Aldrich), β-mercaptoethanol (100 μM; Sigma-Aldrich) and Strep-Pen (100 mg/mL; Gibco). After 3 days, the EB aggregates were plated on a fibronectin pre-coated petri dish and the medium was changed to N2B27 medium (Takara). After 4 days, the ratio of GFP-positive cells was determined using a FACS machine (Arail III). Harvested NSCs were further cultured in N2B27 medium supplemented with EGF (20 ng/mL; Peprotech) and bFGF (20 ng/mL; Peprotech) for proliferation.

2.9. Differentiation of mouse central serotonergic neurons

Differentiation of mouse central serotonergic neurons was carried out on a small scale, as previously described (Barberi et al., 2003). Briefly, after monolayer differentiation for 5 days, the cells were trypsinized and counted. Cells (2×10^4) were seeded onto polyornithine (Sigma-Aldrich) and laminin-coated 12-mm glass coverslips. The specific conditions for serotonergic neurons were: days 5–8, N_2B_{27} + FGF4 (100 ng/mL) + SHH (500 ng/mL); days 8–11, N2 medium (N2) + bFGF (10 ng/mL) + FGF8 (100 ng/mL) + SHH (500 ng/mL); days 11–14, N2 + ascorbic acid (200 μM) + BDNF (20 ng/mL).

2.10. Collection of samples for measurement of serotonin release and quantification of serotonin using HPLC

To detect serotonin released into the medium during neuronal differentiation, we collected medium from the culture every day. The collected media were stored at −80 °C before analysis (Lu et al., 2016). Extracellular serotonin concentrations were assayed by HPLC. Culture media (300 mL) from the samples were acidified with trichloroacetic acid (TCA) (300 mL, 0.612 M). The samples were mixed thoroughly by vortexing for 30 s and then incubated for 10 min at room temperature. The samples were then centrifuged (5000 rpm, 5 min) and a sample (50 μL) of the supernatant was injected onto a 1260 HPLC system (Agilent Technologies), equipped with a DAD detector, FLD detector, and a C18 column (100 mm, 4.6 mm; Agilent Technologies). The mobile phase was KH_2PO_4 (0.1 M) and glacial acetic acid (0.245 M) in water (pH = 4.0). ChemStation software (Agilent Technologies) was used to control the HPLC system and to collect data. The amount of serotonin in each sample was calculated using calibration curves of serotonin standards at different concentrations.

2.11. Determination of molecular weight of "serotonin" peak identified by HPLC

To confirm that the peak identified by HPLC was serotonin, an Orbitrap Fusion mass spectrometer (MS) was used to compare the molecular weight of this compound with that of an authentic sample of serotonin. A full MS scan was performed using the following conditions: detector, Orbitrap; resolution, 500,000; scan range (m/z), 100–500; RF lens, 60%; AGC target, 2.0 e5; and maximum injection time, 100 ms.

2.12. Electrophysiology

Cells were seeded onto poly-D-lysine/laminin-coated 35 mm culture dishes. Whole-cell patch-clamp recordings were made for mouse ESC-derived serotonergic neurons at 2 weeks. The microscope (Nikon) was fitted with a 40× objective lens, configured for DIC. The bath solution contained NaCl (119 mM), $NaHCO_3$ (26.2 mM), glucose (11 mM), KCl (2.5 mM), $CaCl_2$ (2.5 mM), $MgCl_2$ (1.3 mM), and K_2HPO_4 (1 mM). Recording pipettes were filled with an intracellular solution containing KCl (130 mM), NaCl (10 mM), $MgCl_2$ (2 mM), HEPES (10 mM), EGTA (0.5 mM), and $CaCl_2$ (0.16 mM), adjusted to pH 7.2 with KOH (1 M). Whole-cell currents were recorded in voltage-clamp mode, with a basal holding potential of −70 mV. Voltage steps from −90 to +30 mV were delivered at 10 mV increments. TTX (100 nM) was added and data were analyzed using PClampfit 10.2 software.

2.13. Tail suspension test in mice

Fifteen male and fifteen female mice were randomly divided into three groups. The first group (normal control group), received intraperitoneal injections of normal saline 24, 5 and 1 h before the test. The second group (fluoxetine group) received intraperitoneal injections of fluoxetine (2 mg/kg, dissolved in the same volume of saline) 24, 5, and 1 h before the test. The third group (Compound 3 group) received intraperitoneal injections of Compound 3 (2 mg/kg, dissolved in the same volume of saline) 24, 5, and 1 h before the test. Both fluoxetine and Compound 3 were dissolved in normal saline at a concentration of 1 mg/mL.

To measure depression times, the mice were suspended by their tails in a 30 cm × 30 cm × 30 cm carton, using a tape attached about 2 cm from the end of the tail. Initially, the animals struggled to overcome the unusual posture, but, after a time, they demonstrated intermittent immobility, indicative of despair. The suspension test lasted for 6 min and the total time spent immobile during the last 4 min was recorded as the despair time. Experimental data are provided in Additional file 7: Movie S1 and Additional file 2: Table S2.

2.14. Forced swimming test in mice

Fifteen male and fifteen female mice were randomly divided into three groups and drugs were administered as described for the tail suspension test. To measure despair time, the mice were placed into glass cylinders, (20 cm high, and 14 cm in diameter), with one mouse per cylinder, separated by paperboard. The depth of the water was 10 cm and the water temperature was 25 °C. The forced swimming time lasted for 6 min and total periods of immobility in the last 4 min were recorded as the despair time. Immobility meant that the mouse had stopped struggling in the water and was floating, with only small limb movements to keep the head above the water. Experimental data are provided in Additional file 8: Movie S2 and Additional file 3: Table S3.

3. Results

3.1. Extraction of phloroglucinol derivatives from Hypericum longistylum

The air-dried aerial parts of *Hypericum longistylum* were extracted with methanol (MeOH)under reflux. The residue was dissolved in H_2O and extracted with petroleum ether. The petroleum ether soluble portionwas chromatographed on a silica gel column, using a petroleum ether-acetone step gradient system. We divided the extractive into 10 initial fractions according to the ratio of the thin layer shift roughly. About 20 compounds were isolated from the 10 fractions and only the compounds obtained from Fr. 4, Fr. 5 and Fr. 6 may be active. Four compounds were finally isolated after further purification by medium pressure liquid chromatography (MPLC) and high-performance liquid chromatography (HPLC) (Fig. 1a). The structures of these compounds were elucidated by extensive 1-dimensional (1D) and 2D nuclear magnetic resonance (NMR) spectroscopy experiments, and the absolute configurations were established by comparing experimental electronic circular dichroism (ECD) spectra with those calculated using the time-dependent density functional theory (TDDFT) method (Fig. 1b and Additional file 1: Fig. S1). The results indicated Compounds 1, 2, 3, and 4 were all polycyclic phloroglucinols. Compounds 1 and 2 shared the same skeletal structure, and compounds 3 and 4 also shared a skeletal structure (Fig. 1b). The absolute configurations of the four compounds were, however, different from each other. The absolute stereochemistry of the compounds was: Compound 1 (2S, 3R, 4S, 6S, 11S), Compound 2 (2S, 3R, 4S, 6S, 11R), Compound 3 (2S, 3R, 4S, 6S, 18S), and Compound 4 (2S, 3R, 4S, 6S, 18R) (Fig. 1c).

3.2. Phloroglucinol compounds promoted neural precursor differentiation of ESCs in vitro

To explore possible mechanisms of depression, we investigated the effects of the four phloroglucinol derivatives on neural differentiation of ESCs *in vitro*. Existing methods of neural differentiation are empirical and all share the shortcoming that the efficiency of differentiation is not stabilized (Ying et al., 2003; Brustle et al., 1999; Kawasaki et al., 2000). To solve this problem, a modified method was established and shown to have an efficiency of 30–40% (Li et al., 2018).Briefly, viable ESCs with a Pax6-GFP reporter system were selected and cultured with embryoid body (EB) medium in non-coated petri dishes for 3 days. The EB aggregates were then plated on fibronectin pre-coated petri dishes with N2B27 medium. After 4 days, GFP positive cells were analyzed by fluorescence-activated cell sorting (FACS). Pax6 is a marker gene for neural precursor cells (NPCs) and the efficiency of natural differentiation could be monitored by green fluorescence since the GFP gene was pax6 promoter-derived (Fig. 2a). In preliminary experiments, the green fluorescence changed dramatically on the seventh day (data not shown), and was suitable as a time window to evaluate the efficiency of neural differentiation. The effects of compounds 1, 2, 3, and 4 on neural differentiation were determined by adding compound (10 μM) to the culture medium on the second day of differentiation. Compound 3 improved neurogenesis (Fig. 2b) to a greater extent than the other compounds (Additional file 5: Fig. S2a–d). Statistical results showed that Compound 3 increased GFP-positive cells by as much as 65.7%. Immunofluorescent staining of these cells indicated that they expressed NPC markers, Sox1, Nestin, and Pax6 (Fig. 2c).

To further explore the role of Compound 3 on neurogenesis, we added different concentrations into the culture mediums. The efficiency of neurogenesis increased markedly with increasing dose, as determined by expression of neural specific marker genes (Fig. 2d). Interestingly, although the number of Pax6-GFP positive cells increased (Fig. 2b), the average expression level of Pax6 did not change much, which indicated that Compound 3 can promote the differentiation of the Pax6 positive, not the expression level of Pax6A. CCK-8 assay, based on succinic dehydrogenase activity in mitochondria, was used to evaluate the metabolic activity of neural precursor cells differentiated from mouse ESCs (mESCs). Compound 3 did not show significant cytotoxicity over the concentration range 2.5–10 μM, but cell viability decreased significantly when the concentration reached 20 μM (Fig. 2e). The IC_{50} value of Compound 3 was ~ 13.04 μM (Additional file 5: Fig. S2 g). Taken together, the results showed that the optimal concentration of Compound 3 on neural differentiation was 10 μM and this concentration was thus used for further experiments. Retinoic acid (RA) is a well-known growth factor that regulates differentiation of ESCs, especially neural differentiation (Ying et al., 2003). To demonstrate the role of Compound 3 playing in neural differentiation, we induced 10 μM Compound 3 into the differentiation medium of ESCs and with 1 μM RA in the control group. The results suggested that Compound 3 treated group could derive many neural rosettes with good morphology compared with RA treated group (Fig. 2f). The differentiation efficiency of Compound 3 treated group was higher than that of RA treated group by Pax6-GFP positive cells analysis on the 7th differentiation day (Fig. 2g). According to DRAQ7 staining of differentiated cells on the 7th day, there were about 59.3% live cells in 10 μM Compound 3 group, and 56.3% live cells in 1 μM RA group (Fig. 2h). It meant the Compound 3 showed less cytotoxicity than RA in the differentiation. We next investigated the effect of Compound 3 on neural differentiation of human ESCs (hESCs) and found that it had a similar role as a neural trigger. When treated with Compound 3 in neural stem cell (NSC) medium, the cellular morphology of hESCs changed more drastically than in the absence of the compound (Additional file 5: Fig. S2e), as determined by the expression of neural specific markers, Nestin, Pax6, BLBP, and Dcx (Additional file 5: Fig. S2f).

3.3. Compound 3 facilitated serotonergic neuronal differentiation in vitro

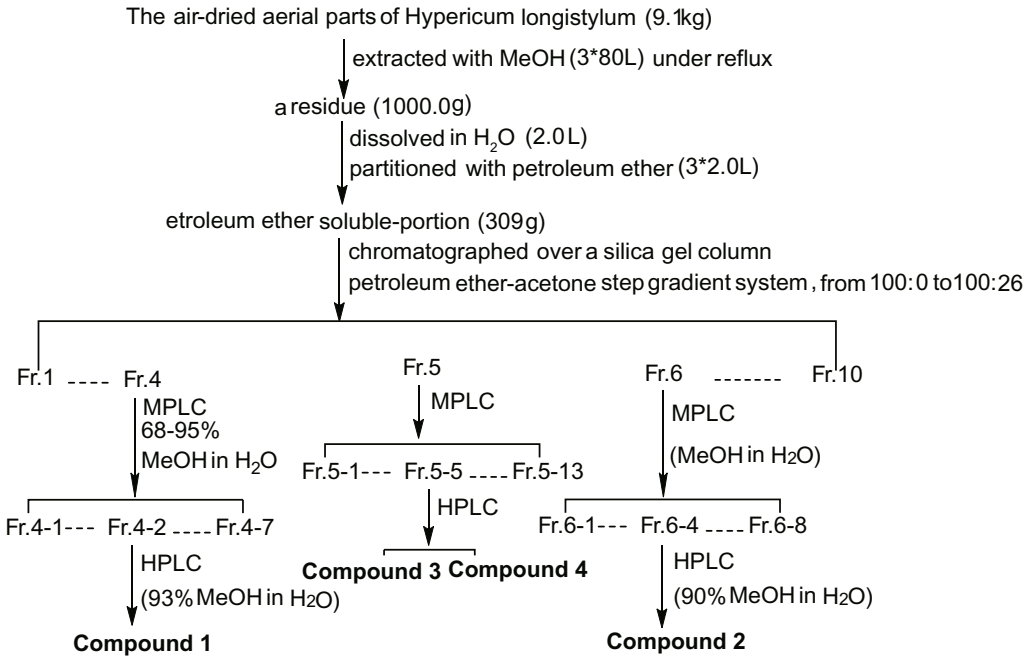
Compound 3 (Fig. 1c) is a phloroglucinol derivative, extracted from *Hypericum longistylum*, which has been reported to have anti-depressive activity. Because depression is known to be closely associated with the function of serotonergic neurons, we evaluated the effects of Compound 3 on serotonergic neuronal differentiation. Serotonergic neurons were generated using an optimized protocol. Briefly, ESCs were for monolayer differentiation in N2B27 medium for 5 days; the medium was then replaced with N2B27 medium, supplemented with fibroblast growth factor-4 (FGF4) and sonic hedgehog (SHH), and the cells were cultured for another 3 days; the cells were then re-plated in N2 medium, supplemented with basic fibroblast growth factor (bFGF), fibroblast growth factor-8 (FGF8), and SHH, and cultured for another 3 days; the medium was then replaced with N2 medium, supplemented with ascorbic acid and brain-derived neurotrophic factor (BDNF), and the cells were cultured for a further 3 days. On day 14, there was a significant increase of serotonergic neurons in cells treated with Compound 3 compared with control cells (Fig. 3a). Similar results were observed by immunofluorescent staining of serotonin transporter (SERT) (Fig. 3b) and qPCR (Fig. 3c). Whole-cell patch-clamp recordings were also carried out on the serotonergic neurons. Some cells exhibited spontaneous action potentials, which could be blocked by tetradotoxin (TTX), a specific inhibitor of sodium ion channels. When recording in the voltage-clamp mode, we observed inward fast inactivating currents and outward currents, which may correspond to the opening of voltage-dependent Na + and K + channels, respectively. The inward current could be blocked and recovered by addition and removal of TTX. Whole-cell potassium sodium current was recorded to determine the functional membrane electric potential of the neurons (Fig. 3d). HPLC was used to measure the amount of serotonin released during differentiation and confirmed the function of the serotonergic neurons. Medium was collected at five time points after differentiation of the cells, with or without Compound 3. On days 9, 11, and 13, the amount of serotonin released by cells treated with Compound 3 was significantly higher than that released by the control group (Fig. 3e and Additional file 6: Fig. S3). These results showed that Compound 3 not only improved the differentiation efficiency from ESCs to serotonergic neurons but also increased the amount of serotonin released. To confirm that the peaks identified by HPLC were, indeed, serotonin, their molecular weight was determined using an Orbitrap Fusion mass spectrometer. The molecular weight was found to be 177.10243, equal to a serotonin standard (Fig. 3f).

3.4. Compound 3 reduced depression-like behaviors in mice

Compound 3 was compared with fluoxetine, a marketed antidepressant drug, in models of depression in mice. Sixty mice were randomly divided into six groups, with five males and five females in each group. Three groups of mice were subjected to the tail suspension test and three groups of mice were subjected to the forced swimming test. For the tail suspension test, the groups were injected intraperitoneally with normal saline, fluoxetine (2 mg/kg), or Compound 3 (2 mg/kg). No adverse reactions were observed in mice in any of the groups prior to recording of data. Fluoxetine and Compound 3 both shortened the time spent motionless in the tail suspension (Additional file 2: Table S2 and Fig. 4a), with no significant differences between the two groups. The same dosing regimen was used in the forced swimming test. Compared with normal saline, both fluoxetine and Compound 3 reduced the swimming immobility time (Additional file 3: Table S3). All of the experiments were recorded (Additional file 7: movie S1 and Additional file 8: movie S2).

In summary, we extracted four natural products from the air-dried aerial parts of *Hypericum longistylum* and explored their anti-depressive effects using a pax6-GFP reporter system. We identified Compound 3 as the active ingredient in neurogenesis and serotonergic neuronal

a



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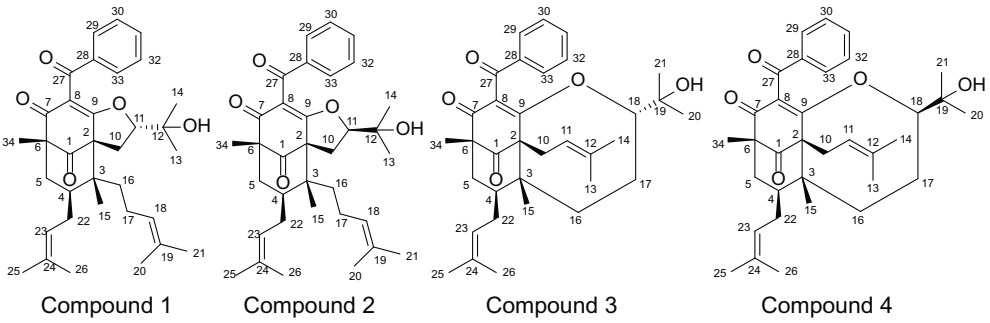

c

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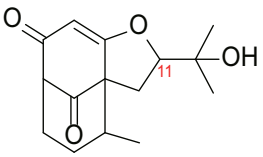
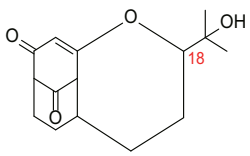
Skeleton [2,3]				
Absolute configuration	2S, 3R, 4S, 6S, 11S	2S, 3R, 4S, 6S, 11R	2S, 3R, 4S, 6S, 18S	2S, 3R, 4S, 6S, 18R

Fig. 1. Separation and identification of natural compounds.
(a) Schematic overview of strategy to isolate compounds from air-dried aerial parts of *Hypericum longistylum*. (b) Structures of compounds 1–4. (c) Comparison of molecular properties of compounds 1–4.

differentiation and demonstrated that this compound could reduce depression-like behaviors in mice (Fig. 4b).

4. Discussion

In the present study, we have demonstrated that Compound 3, extracted from *Hypericum longistylum*, significantly facilitated generation of neural stem cells and serotonergic neuron differentiation of ESCs. This is the first example of a compound extracted from a Chinese herb that regulates neural lineage specification. *Hypericum longistylum* is a valuable medicinal plant that grows abundantly in China and has been proven to be useful as an antidepressant. In this study, we not only identified the primary active component as a phloroglucinol compound but also assessed its effect on neural regeneration using *in vitro*

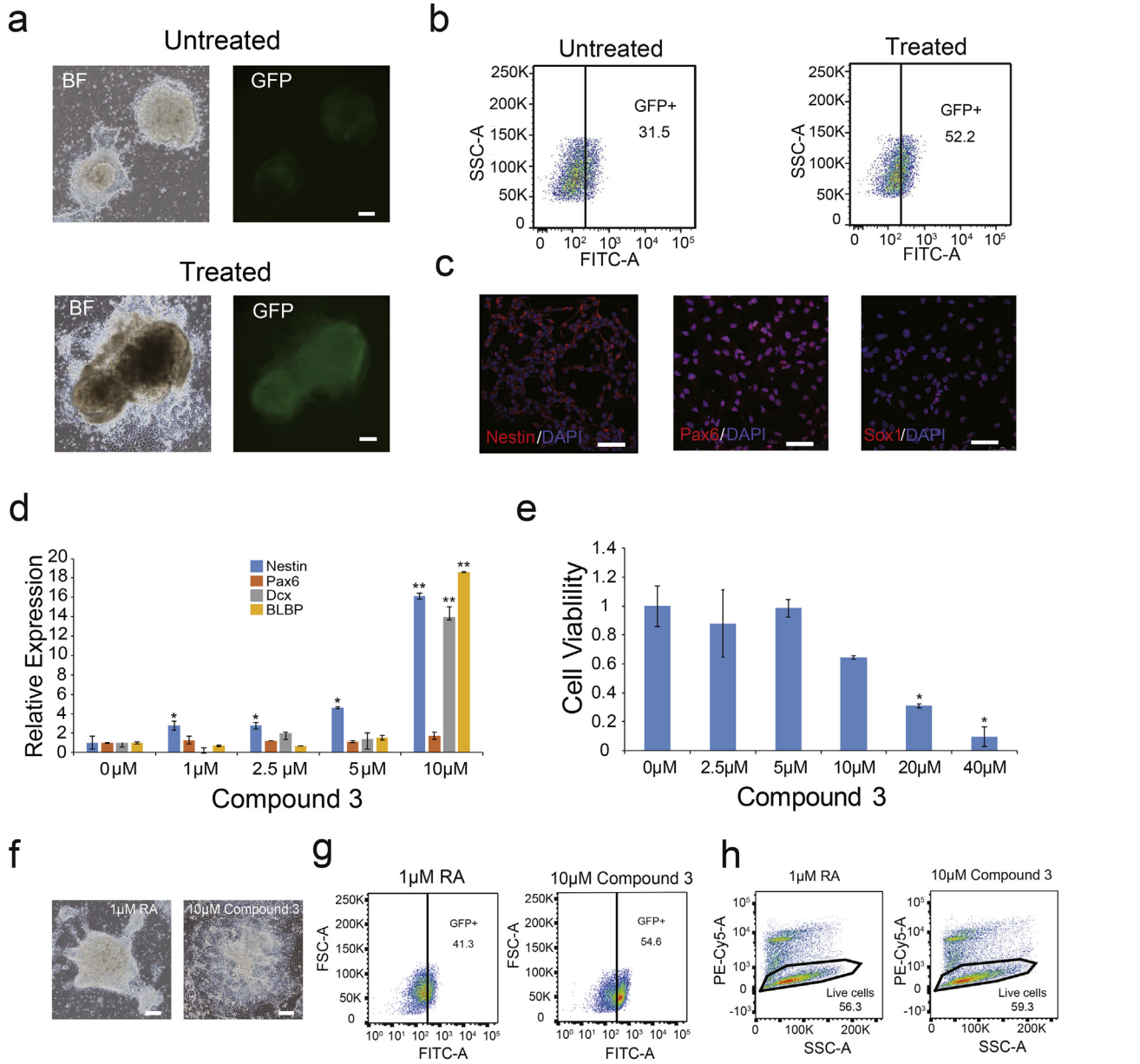


Fig. 2. Compound 3 facilitated neurogenesis.
(a) Bright field (BF) and fluorescence images of pax6-GFP bi-allele knock-in cell line cultured in neural specification medium in the presence or absence of Compound 3 on day 7; scale bar = 100 μm. (b) FACS analysis of GFP-positive cells after differentiation in the presence or absence of Compound 3 on day 7. (c) Immunofluorescence analysis of NPC-specific markers in GFP-positive cells sorted on day 7; Nestin (red), Sox1 (red), and Pax6 (red). DAPI (blue) was used for staining of nuclei; scale bar = 100 μm. (d) RT-PCR analysis of NSC marker genes (*Pax6* and *Nestin*) and neuron marker genes (*Dcx* and *BLBP*) on day 7 of cells differentiated in the presence of different doses of Compound 3. (e) Viability of ESCs in the presence of different doses of Compound 3 compared with viability of ESCs in the absence of Compound 3. (f) Cellular morphology of cell line cultured in neural specification medium with 1 μM RA and 10 μM Compound 3 on day 7. Scale bar, 100 μm. (g) FACS analysis of GFP-positive cells after differentiation in the presence of RA and Compound 3 on day 7. (h) A DRAQ7 staining analysis was conducted to detect the cell viability with 1 μM RA and 10 μM Compound 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiments. Compound 3 improved the efficiency of neural stem cell differentiation and also promoted the generation of serotonergic neurons. Injection of Compound 3 was also shown to reduce depression-like behaviors of mice in a stressful environment.

Biologically active compounds that are not genotoxic have been widely used in cell reprogramming (Hou et al., 2013; Li et al., 2017) and trans-differentiation research (Li et al., 2015). We compared the effects of our phloroglucinol compounds with those of RA on neural differentiation of ESCs *in vitro*. ESCs treated with Compound 3 yielded more neural stem cells than control cells treated with RA (Fig. 2f and g), and also showed better ability to survive the DRAQ7 staining process (Fig. 2h). Compound 3 is a novel compound derived from *Hypericum longistylum* that efficiently induced neural differentiation of ESCs. During neuronal differentiation, ESCs treated with Compound 3 also produced more serotonergic neurons than non-treated control cells, resulting in higher levels of 5-HT. This could be the main reason why mice injected with Compound 3 showed more normal behaviors in stressful environments. A possible mechanism for the beneficial effects of *Hypericum longistylum* on depression could be that the phloroglucinol compounds induce neural regeneration *in vivo*.

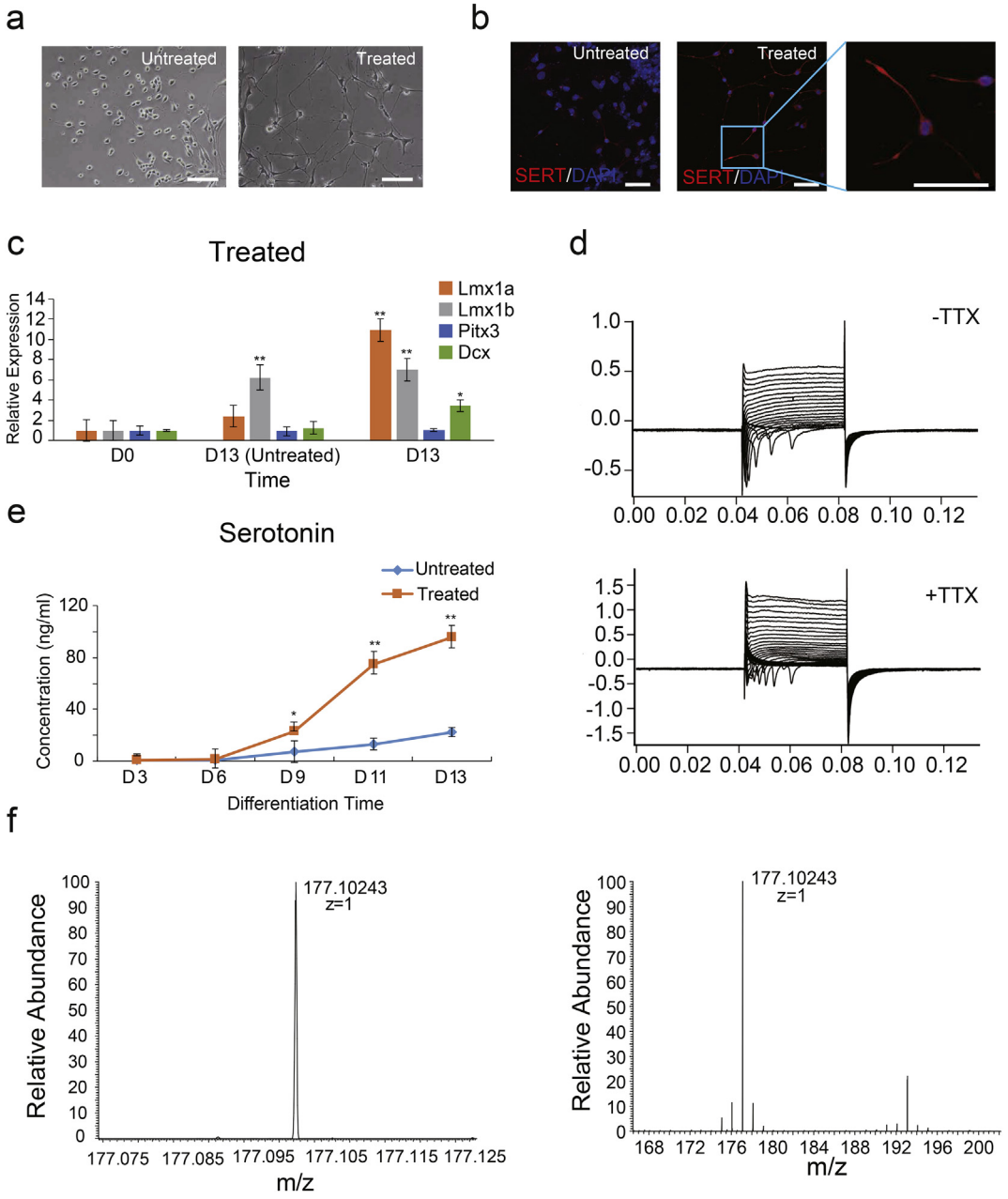


Fig. 3. Compound 3 drives differentiation of ESCs to the serotonergic phenotype.
(a) Bright field (BF) images showing cellular morphology on day 13 of differentiation without (left) or with (right) Compound 3. Scale bar = 100 μm. (b) Immunofluorescence staining with serotonergic neuron-specific maker SERT (red). DAPI (blue) was used for staining of nuclei. Scale bar = 25 μm. (c) RT-PCR analysis of serotonergic neuron marker genes (*Lmx1a*, *Lmx1b*, *Pitx3* and *Dcx*) in differentiated cells with and without Compound 3 on day 13. (d) Electrophysiological properties of derived serotonergic neurons with Compound 3. Representative voltage-clamp recordings in response to increasing voltage pulses from neurons. Top, −TTX; Bottom, +TTX. (e) Statistical analysis of serotonin levels in culture supernatants collected on days 3, 6, 9, 11, and 13 during differentiation. (f) Mass spectroscopic analysis of molecular weight of compound identified as "serotonin" peak by HPLC. Left, serotonin standards; Right, Compound 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusions

Overall, we found that the key components of *Hypericum longistylum* were phloroglucinol compounds, which had favorable effects on neural differentiation and the production of serotonergic neurons. Compound 3 is the first compound extracted from a Chinese herb with specific effects on neurons and should be valuable both for the treatment of depression and for mechanistic studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.07.003.

Ethical Approval and consent to participate

All mouse experiments were performed in accordance with the relevant institutional and national guidelines and regulations; the experiments were approved by the Animal Care and Use Committee of Nankai University, and all experiments conform to the relevant regulatory standards.

Consent for publication

Not applicable.

a

Analysis result of mouse behavior when in danger ($\overline{X}$ ± S, n=10)

Group	mouse tail suspension immobility time		mouse forced swimming immobility time	
	Dosage (mg•Kg^{-1})	Time (s)	Dosage (mg•Kg^{-1})	Time (s)
Normal saline	2	123.1 ± 13.08	2	121.4 ± 20.74
Fluoxetine	2	73.5 ± 24.94*	2	62.1 ± 14.46*
Compound 3	2	90.8 ± 20.47*	2	94.4 ± 26.21*

***Compared to the saline group, P<0.01**

b

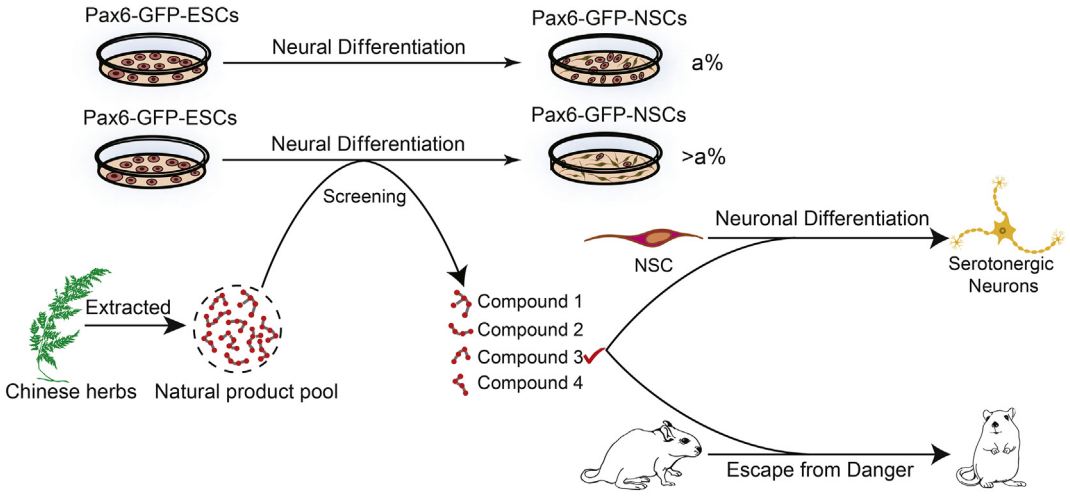


Fig. 4. Compound 3 improved mouse behaviors under stress.
(a) Immobility times in mouse tail suspension test and forced swimming test. A *t*-test showed that immobility times were significantly different between the groups treated with Compound 3 or fluoxetine and the control group ($p < 0.01$). Both tests lasted for 6 min and the time spent immobile (despair time) was recorded during the last 4 min. (b) Graphic model showing role of Compound 3. Compound 3 facilitated neurogenesis and serotonergic neuronal differentiation *in vitro*. The compound also reduced depression-like behaviors of mice placed in a stressful position.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.S. designed and guided the study; L.S., Y.Q.G. and Y.Y. supervised it. H.S.W. and W.H.Z. performed the experiments. H.S.W., W.H.Z., X.R.C., Y.N.L., X.L., Q.G., and Z.Y.M. collected the data. L.S., H.S.W., and W.H.Z. wrote the manuscript.

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References

Barberi, T., Klivenyi, P., Calingasan, N.Y., Lee, H., Kawamata, H., Loonam, K., Perrier, A.L., Bruses, J., Rubio, M.E., Topf, N., Tabar, V., Harrison, N.L., Beal, M.F., Moore, M.A., Studer, L., 2003. Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat. Biotechnol. 21, 1200–1207.

Brambilla, F., Dalle Grave, R., Calugi, S., Marchesini, G., Baroni, S., Marazziti, D., 2010. Effects of cognitive-behavioral therapy on eating disorders: neurotransmitter secretory response to treatment. Psychoneuroendocrinology 35, 729–737.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., McKay, R.D., 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285, 754–756.

Cao, X., Yang, X., Wang, P., Liang, Y., Liu, F., Tuerhong, M., Jin, D.Q., Xu, J., Lee, D., Ohizumi, Y., Guo, Y., 2017. Polycyclic phloroglucinols as PTP1B inhibitors from Hypericum longistylum: structures, PTP1B inhibitory activities, and interactions with PTP1B. Bioorg. Chem. 75, 139–148.

Choi, S., 2003. Nefazodone (Serzone) withdrawn because of hepatotoxicity. CMAJ 169,

1187.
Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., Liu, K., Ge, J., Xu, J., Zhang, Q., Zhao, Y., Deng, H., 2013. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341, 651–654.
Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Nishikawa, S.I., Sasai, Y., 2000. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 28, 31–40.
Li, M., Pevny, L., Lovell-Badge, R., Smith, A., 1998. Generation of purified neural precursors from embryonic stem cells by lineage selection. Curr. Biol. 8, 971–974.
Li, G.Y., Ueki, H., Yamamoto, Y., Yamada, S., 2006. Association between the scores on the general health questionnaire-28 and the saliva levels of 3-methoxy-4-hydroxyphenylglycol in normal volunteers. Biol. Psychol. 73, 209–211.
Li, X., Zuo, X., Jing, J., Ma, Y., Wang, J., Liu, D., Zhu, J., Du, X., Xiong, L., Du, Y., Xu, J., Xiao, X., Wang, J., Chai, Z., Zhao, Y., Deng, H., 2015. Small-molecule-driven direct reprogramming of mouse fibroblasts into functional neurons. Cell Stem Cell 17, 195–203.
Li, X., Liu, D., Ma, Y., Du, X., Jing, J., Wang, L., Xie, B., Sun, D., Sun, S., Jin, X., Zhang, X., Zhao, T., Guan, J., Yi, Z., Lai, W., Zheng, P., Huang, Z., Chang, Y., Chai, Z., Xu, J., Deng, H., 2017. Direct reprogramming of fibroblasts via a chemically induced XEN-like state. Cell Stem Cell 21, 264–273 (e267).
Li, Y., Li, X., Wang, H., Gao, Q., Zhang, J., Zhang, W., Zhang, Z., Li, L., Yu, Y., Shuai, L., 2018. CRISPR/Cas9-edited Pax6-GFP reporter system facilitates the generation of mouse neural progenitor cells during differentiation. J. Genet. Genomics 45, 277–280.
Lu, J., Zhong, X., Liu, H., Hao, L., Huang, C.T., Sherafat, M.A., Jones, J., Ayala, M., Li, L., Zhang, S.C., 2016. Generation of serotonin neurons from human pluripotent stem cells. Nat. Biotechnol. 34, 89–94.
Stahl, S.M., 2014. Mechanism of action of the SPARI vilazodone: serotonin 1A partial agonist and reuptake inhibitor. CNS Spect. 19, 105–109.
Steidtmann, D., Manber, R., Arnow, B.A., Klein, D.N., Markowitz, J.C., Rothbaum, B.O., Thase, M.E., Kocsis, J.H., 2012. Patient treatment preference as a predictor of response and attrition in treatment for chronic depression. Depress. Anxiety 29, 896–905.
Willner, P., Scheel-Kruger, J., Belzung, C., 2013. The neurobiology of depression and antidepressant action. Neurosci. Biobehav. Rev. 37, 2331–2371.
Xue, T., Roy, R., 2003. Studying traditional Chinese medicine. Science 300, 740–741.
Yang, D., Li, T., Xu, M., Gao, F., Yang, J., Yang, Z., Le, W., 2014. Graphene oxide promotes the differentiation of mouse embryonic stem cells to dopamine neurons. Nanomedicine (London) 9, 2445–2455.
Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., Smith, A., 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat. Biotechnol. 21, 183–186.