The Role for GABAergic Interneurons Subtypes in the Antidepressant Efficacy of Traditional Chinese Medicine Formula Lily Bulb and Rehmannia Decoction

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Research

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Abstract

Background

Depression is associated with reduced γ-aminobutyric acid (GABA) and alterations of GABAergic interneurons in medial prefrontal cortex (mPFC), which can be normalized by traditional Chinese medicine formula lily bulb and Rehmannia decoction (LBRD) standard decoction. However, the impact of GABAergic interneurons subtypes including parvalbumin (PV), somatostatin (SST), and vasoactive intestinal peptide (VIP), in depressive episodes as well as in the LBRD standard decoction antidepressant effects remains to be elucidated.

Methods

We assessed the effects of LBRD standard decoction intervention on the expression of GABA associated miRNA/mRNA and GABAergic subtype-specific markers in chronic stress induced depression by western blot and qRT-PCR. The lncRNA/miRNA/GABA regulatory axis was verified by luciferase reporter assay, RNA immunoprecipitation, RNA pull-down assay and then detect its changes in LBRD standard decoction administration with use of immunofluorescence staining and RNA-fluorescence in situ hybridization (FISH).

Results

In the current study, we found that LBRD standard decoction exerted antidepressant effects based on the results of behavioral tests. Meanwhile, LBRD also revised the reduced GABA levels in depression by increasing the expression of lncRNA Neat1 and Malat1, as well as decreasing miRNA-144-3p and miRNA-15b-5p. Moreover, the expression of SST mRNA and protein in the mPFC tissues from depression group, was significantly lower than those in the control cases, while LBRD standard decoction treatment revised these changes. Whereas, neither chronic stress nor treatment can obviously change the level of PV and VIP mRNA and protein expression. In the SST-positive neurons of mPFC tissues, treatment with LBRD standard decoction resulted in the elevation of Gad-67, VGAT, GAT-3 and an reduction of miRNA-144-3p expression.

Conclusion

Taken together, these findings suggest that SST interneurons may be served as a preferentially vulnerable GABAergic neuronal subtype in depression and LBRD standard decoction antidepressant activities potentially relate to against the SST cells deficits by regulating the miRNA-144-3p mediated GABA synthesis, release.

1. Background

Depression is a highly heterogeneous disease with diverse etiological causes [1]. Currently, antidepressant drugs characterized by single-target and single-factor therapy are the preferred choice to cope with depression [2]. However, those agents are effective only for partially depressed patients and bring numerous undesirable side effects including chronic sexual dysfunction, insomnia, acute nausea and headaches, and also increase the risk of cardiovascular, urinary and digestive dysfunction illness etc. [3].

In contrast, traditional Chinese medicine (TCM) characterized by syndrome differentiation, has a long history and the potential to prevent and treat depression [4]. Especially, some classical formulas by ZHANG Zhongjing from the ancient time has unique advantages in the treatment of depression [5]. One classical formula by him, Lily Bulb and Rehmannia Decoction (LBRD), which is comprised of lily bulb, raw rehmannia root juice and spring water, has been used clinically by countless doctors in the treatment of various types of depression with definite curative effects and few side effects [6]. In recent years, great progress achieved in basic studies and clinical practice, promote a better understanding of the underlying neurobiological basis of LBRD as a novel antidepressant agent. It is believed a multi-component with multi-target mechanism might be the essential for which LBRD assists depressed patients to achieve the yin-yang balance of the body [7–9].

Accumulating evidences from postmortem studies, brain imaging, as well as chronic stress models, demonstrate dysfunction of inhibitory γ-aminobutyric acid-ergic (GABAergic) interneuron, as well as excitatory glutamate neurons contributes to depression-related behavior [10]. Notably, lower GABA levels have been repeatedly observed in the medial prefrontal cortex (mPFC) from depressive patients and depression-like behavior animal model [11]. More importantly, GABA levels increase occurs in the effective treatment for depression by medications, as well as by classical TCM formula intervention [12]. Furthermore, reductions of GABA markers are also found in bipolar disorder [13] and schizophrenia [14], suggesting that GABA dysfunction is a vulnerability factor and may be served as potential therapy target for affective disorder [15]. In line with these studies, we have found that GABA deficit is related to the depression-like behaviors induced by chronic unpredictable mild stress (CUMS), and can be revised by LBRD standard decoction treatment. However, the mechanisms underlying the LBRD-mediated regulation of GABA have not yet been investigated at the molecular level.

At the epigenetic level, competitive endogenous RNA (ceRNA) network regulating transcript RNAs such as long noncoding RNAs (lncRNAs), can act as natural miRNA sponges to inhibit miRNA functions and modulate mRNA expression [16]. Until now, ceRNA regulation network mediated by lncRNA-miRNA-mRNA have emerged as a therapeutic target in depression [17–23]. We have previously constructed the GABA miRNA/mRNA network in which glutamate decarboxylase-67 (Gad-67) and vesicular GABA transporter (VGAT) can be directly regulated by miR-144-3p, miR-15b-5p and miR-879-5p [24, 25]. Interestingly, lncRNAs are highly expressed in the brain and involved in important neurobiological process, including neurotransmitter synthesis and transmission, neurogenesis, synaptic efficiency, and neural plasticity [26,

[27]. In this regard, lncRNAs may play important roles in the pathogenesis of depression and anxiety, potentially representing a new class of therapeutic targets that can be exploited for disease treatment. Whereas, lncRNA-mediated sponge regulatory network underlying GABA deficit in depression remain inconclusive.

GABAergic interneurons are identified by their expression of specific reports for calcium-binding protein parvalbumin (PV, ~40%), neuropeptide somatostatin (SST, ~30%), and ionotropic serotonin receptor 5HT3aR (~30%), including vasoactive intestinal peptide (VIP)-expressing interneuron [28]. Postmortem studies of depressed subjects demonstrated the alterations of cortical GABAergic interneurons subpopulation, notably SST, and less consistently PV and VIP [29–31]. The results from SST and PV markers studies have been mixed in rodent depression model, with reports that result in a selective reduction of SST-positive interneurons in the hippocampus and mPFC tissues with no change in PV [32], and other reports that long-term chronic mild stress decreases PV, but not SST, in mPFC tissue of susceptible rats [33]. Another recent study shows that CUMS for five weeks can decrease the protein level of Gad-67, as well as SST, with a similar trend for PV in the mPFC tissue [34]. In contrast to these effects of stress, evidence supports a role for SST interneurons in the antidepressant efficacy of current treatments and in experimental manipulations with fast-acting antidepressant-like activity [35–37]. Although it has been implicated that GABAergic interneurons could be the target in depression therapy, whether LBRD standard decoction treatment regulating GABAergic interneuron subpopulations remain unknown.

Hence, we hypothesized that LBRD standard decoction could improve depressive behavior by increasing GABA concentrations via ceRNA networks and resorting the GABAergic interneuron subpopulations homeostasis, and designed the current study aiming to validate our hypothesis and uncover novel pharmacological mechanism for it.

2. Methods

2.1 Preparation of LBRD standard decoction

LBRD standard decoction is consists of 400 g of fresh lily bulbs from Shennongjia (Hubei Province, China) and 400 g of fresh raw rehmannia root from Jiaozuo (Henan Province, China) and its preparation procedures was referred to our previous study [6, 38]. Ultra-high performance liquid chromatography quadrupole time of flight tandem mass spectrometry (UHPLC-Q-TOF/MS) was applied to detect the phytoconstituents of LBRD standard decoction and its constituent herbs (Dionex Ultimate 3000 HPLC Plus Focused coupled to an LTQ/Orbitrap MS system, Thermo Scientific, United States). To assure the quality of LBRD standard decoction, the fingerprinting of the chemical marker (verbascoside and catalpol) was measured by HPLC for quality monitoring.

2.2 Animals and Treatment

Male C57BL/6J juvenile mice with 4 weeks old (16-18 g) were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and maintained in plastic cages in a temperature of 22–25°C and a humidity of 40-70% under a 12-h light/dark cycle, fed with water and food *ad libitum*. After a one-week acclimatization period, mice were randomly divided into the following four groups: control group (no stress), model group (CUMS+distilled water), LBRD group (CUMS+LBRD standard decoction) and fluoxetine group (CUMS+fluoxetine).

The drug administration was conducted one-week after the CUMS modeling (from the second week to the fourth week). The mice in the LBRD group groups were treated with LBRD standard decoction daily with 150 g/kg/d (10 ml/kg body weight) by gavage. The mice in the fluoxetine group were treated with fluoxetine about 2 mg/kg/d with 10 ml/kg body weight (Lilly Suzhou Pharmaceutical Co., Ltd., Suzhou, China). Whereas, mice in control and CUMS groups received an equivalent volume of saline water. Treatments were administered orally to the mice in each group 1 h before modeling. The detailed timeline is shown in Fig. 1A. All animal experiments were approved by Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine (SDUTCM201805311223).

2.3 Chronic unpredicted mild stress modeling and behavioral evaluation

The depression-like model was produced by exposing the mice to chronic unpredicted mild stress (CUMS) according to a previously published protocol [39]. The depression-like behaviors were examined by the sucrose preference test (SPT), forced swimming test (FST) and tail suspension test (TST) as previous described [25, 40].

2.4 Histopathological analysis and biochemical indicators analysis

Sections from mPFC tissues were dewaxed with xylene, passed through an aqueous ethanol series, stained using hematoxylin-eosin (H&E), and observed using microscopy (Olympus Optical Co. Ltd., Tokyo, Japan). Five non-overlapping fields of view were randomly selected in each slice. The integral optical density was determined using the Image-Pro Plus 6.0 system (Media Cybernetic, Silver Springs, MD, USA) for semi-quantitative analysis. The amounts of GABA, glutamate (Glu) and interleukin-1β (IL-1β), IL-10 in mPFC tissue were measured with use of enzyme linked immunosorbent assay (ELISA) kits (Beyotime Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's instructions. Each experiment was conducted in triplicate.

2.5 Quantitative RT-PCR and Western blot analyses

Total RNAs from mPFC tissues extraction were preformed following the instructions of the TRIzol RNA extraction kit (Thermo Fisher, Waltham, MA, USA). Thereafter, the cDNA was generated with use of reverse-transcription kit (Takara, Dalian, China) and amplified using the Power SYBR-Green Premix (Takara, Dalian, China) by the ABI 7500 System (Applied Biosystems, Foster City, CA, USA). The amplification levels of the mRNAs and miRNAs relative to controls were computed based on the 2-ΔΔCt

method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were served as the endogenous control for the normalization of qRT-PCR data. For Western blot analysis, a total protein extraction kit was used to extract protein from mPFC tissues (Beyotime Biotech., Shanghai, China) protein assay. After electrophoresis and electroporation, the converted membrane was blocked with 5% skim milk powder for one hour, washed with TBST buffer and incubated overnight at 4°C with the primary antibody. The electrophoresis membrane was then incubated with the secondary antibody for two hour and washed with TBST buffer. Luminescence was measured by chemiluminescent reagents (Millipore, Billerica, MA, USA) and protein separation membranes were scanned and analyzed using an image analyzer (Bio-Rad, CA, USA). The ratio of the gray value of protein bands to that of internal reference (β-actin) was deemed as the relative protein expression.

2.6 Immunofluorescence staining and RNA-fluorescence in situ hybridization (FISH)

The excised mPFC tissue was finely sliced, fixed and embedded in paraffin. The sample was cut into 5 μm sections, then deparaffinized and rehydrated. After recovery of antigen, sections were treated with H2O2 and blocked with 8% bovine serum albumin (BSA) solution. The sections were then incubated overnight with the primary antibody. The next day, the sections were rinsed and incubated with horseradish peroxidase (HRP) -labeled secondary antibody for 1 hour. The signal was visualized with 3,3'-diaminobenzidine (DAB) (Beyotime, Shanghai, China). Finally, sections were stained with hematoxylin (Sigma, St. Louis, MO, USA), mounted and imaged under a BX51 microscope. RNA-FISH experiments were performed using an RNA in situ hybridization fluorescence kit (Guangzhou Ribo Biotechnology Co., Ltd. Guangzhou, China) according to the manufacturer's instructions. Briefly, tissue pieces were plated on glass slides, fixed in 4% paraformaldehyde for 10 minutes and rinsed with phosphate buffered saline (PBS). The fixed cells were then placed in a hybridization buffer containing a 20 μM FISH probe and reacted overnight at 37 ° C. The next day, the slides were rinsed with PBS and re-stained with 4', 6-diamidino-2-phenylindole (DAPI). Finally, the sample was observed with a confocal fluorescence microscope (Zeiss, LSM710). The miRNA probe was designed and synthesized by Ribo Biotechnology.

2.7 Luciferase reporter assay, RNA immunoprecipitation and RNA pull-down assay

The Starbase (http://starbase.sysu.edu.cn/) was retrieved to predict the binding relationship between lncRNA/miRNA/GABA associated genes axis. The predicted lncRNA sequence containing the miRNAs binding site and mutation binding site was cloned into the pmiRGlo vector to construct the lncRNA-wild type (WT) and corresponding mutant (MUT) reporter vectors. The GABA-related gene-3'UTR reporter (WT and MUT) plasmids were then cotransfected with miRNA mimic or mimic-NC and HEK-293T cells. After 48 hours, cell supernatants were collected and subsequently measured for luciferase activity. This was expressed as the ratio of firefly luciferase activity to reniral luciferase activity. The assay used the MagnaRIP ™ RNA-binding protein immunoprecipitation (IP) kit (Millipore, Billerica, Mass., USA). Briefly, cell extracts were incubated with magnetic beads bound to Anti-Ago2 or anti-IgG for 1 hour at 4 ° C. Next, the

cell lysate was subjected to overnight incubation at 4°C with magnetic beads. Later, purified RNA was subjected to qRT-PCR analysis. The interactions between lncRNAs and miRNAs were examined using RNA pull-down assays with a magnetic RNA-Protein pull down kit (Thermo Fisher, Waltham, MA, USA). Neuro-2a cells underwent lysing by RIPA lysis buffer, incubation at 37°C with Bio-NC, and Bio-lncRNA for gentle rotation overnight at 4°C. The next day, streptavidin-labeled magnetic beads were added and incubated for an additional two hour. Finally, the eluants were collected to detect the expression patterns of miRNAs using qRT-PCR. Each sample was measured in triplicate to get the average.

2.8 Statistical analyses

All experiments were performed at least in triplicate prior to statistical analysis and the results were expressed as mean ± standard error of mean (SEM). Differences between two groups were compared by Student's *t*-test, while the comparison between three or more groups were performed through one-way analysis of variance (ANOVA) followed by Dennett's test in order to evaluate the variance of multiple groups. Spearman's correlations were used to assess the correlations among the expression of miRNAs and target mRNAs from case. $P<0.05$ was considered as statistical significance.

3. Results

3.1 The chemical component profiling and quality control of LBRD standard decoction

The chemical component profiling of LBRD standard decoction was analyzed by UHPLC/Q-TOF/MS (Figure S1), yielding 32 identified compounds including vidarabine, aminoadipic acid, myristic acid, catalpol, verbascoside, coumarin, etc. (Table S1). The alignment of ingredients in LBRD samples matched the corresponding peaks of verbascoside and catalpol by the same HPLC elution system (Figure S2). The mean values of verbascoside and catalpol were 0.009046% and 1.6%, respectively (Table S2), which were in line with the standard qualities of lily bulb and raw rehmannia root in the *Pharmacopoeia of People's Republic of China* (2015 Edition).

3.2 LBRD standard decoction ameliorated depressive-like behaviors

To investigate the effects of LBRD standard decoction on CUMS-induced depressive like behaviors, the SPT, FST and TST were performed after the treatment. Statistical results showed that the model group presented a significant reduction in sucrose consumption and a notable increase in immobile time of FST and TST, compared to that in the control group (**Figure. 1b-d**, both $P<0.001$). After LBRD standard decoction administration, the behavior indicated that the depression status was significantly improved compared to the model group and its active trend was similar to that of the positive drug fluoxetine (**Figure. 1**). The results from ELISA test showed CUMS obviously increased the level of IL-1β and as well as decreased IL-10 concentration in the mPFC of mice compared to those of the normal control group, while LBRD standard decoction obviously retarded the inflammatory response (**Figure. 1e-f**, both $P<0.01$).

Furthermore, LBRD standard decoction treatment reversed imbalances between GABA and Glu neurotransmitter in mPFC tissue exposed to chronic stress (**Figure. 1g-h**, both $P<0.01$). H&E staining found the mPFC tissue from model group exhibited increased pyknosis, neuronal loss, ruptured nuclear membrane, and disappeared nucleolus, in comparison with control mice. However, LBRD standard decoction treatment markedly resisted pathological alterations, suggesting it has protective effect on neuronal morphology (**Figure. 1i**). In summary, LBRD standard decoction treatment can exert the antidepressant effect by rescuing cortical neurons loss induced by the Glu-mediated exitotoxicity and recovering the balance of pro/anti-inflammatory cytokine.

3.3 Effects of LBRD standard decoction administration on the GABA associated genes expression in different GABAergic interneuron subtypes

In order to explore the molecular mechanism underlying LBRD standard decoction effects on GABA level in depression, the gene and protein expression levels related to GABA synthesis (Gad-67), release (VGAT) and uptake (GAT-3) were analyzed by qRT-PCR and western blot analysis. As shown in **Figure. 2a-b**, chronic stress resulted in a decrease in the Gad-67, VGAT and GAT-3 expression in the mPFC tissues, and LBRD standard decoction reversed this change. Considering the heterogeneity of GABAergic interneurons and the receptors, we investigated its subtype-specific vulnerability in CUMS-induced depression and drug treatment. The immunostaining images showed that the fluorescence density and percentage of SST-positive neurons decreased after CUMS exposure in mPFC tissues, while cell numbers increased after treatment with LBRD standard decoction (**Figure. 2C**). However, no clear alterations in either PV or VIP interneuron were detected (**Figure S3**). Meanwhile, the qRT-PCR results showed that the expression of SST mRNA in the mPFC tissues in the model group was lower than that of the mice in the control group. Upon treatment with LBRD standard decoction, the level of SST mRNA was higher than that of model group. Whereas, neither CUMS nor treatment can obviously change the level of PV and VIP mRNA expression (**Figure. 2d)**. The western blot results were consistent with the immunofluorescence and qRT-PCR results (**Figure. 2e**).

Then, we also detected the expression of GABA associated genes in the SST interneuron using the immunofluorescence technique. Semi-quantitative results showed that the expression of Gad-67, VGAT, GAT-3 in SST interneuron from model mPFC tissues was significantly reduced than that of control mice; meanwhile LBRD standard decoction treatments markedly reversed reduction of gene expression related GABA synthesis and uptake (**Figure. 2f-h**). These results reveal that SST interneuron as a GABAergic neuronal subtype that was preferentially vulnerable to stress induced depression, and the antidepressant effect of LBRD standard decoction might be related to modulate the balance of GABAergic interneuron subpopulations.

3.4 LBRD standard decoction modulation ceRNA network by which elevated GABA synthesis, release and uptake associated genes expression

In the miRNA/mRNA regulatory GABA network, Gad-67 and VGAT were directly regulated by seed-like binding regions of miR-144-3p, and miR-15b-5p could be served negative post-regulators by binding to the sites of GAT-3 3'-UTR [25]. After chronic treatment of LBRD standard decoction, the expression levels of miRNA-144-3p and miRNA-15b-5p were significantly reduced in the mPFC tissues compared to control mice (**Figure. 3a**). The linear regression analysis showed that miR-144-3p and miRNA-15b-5p were negatively correlated with the expression of *Gad-67*, *VGAT* and *GAT-3* mRNAs, respectively (**Figure. 3b**).

Emerging evidence has implicated lncRNAs in several molecular biological events through acting as ceRNA for miRNAs. We then used the publicly available algorithm starBase 3.0 to predict the directly interacting miR-144-3p and miRNA-15b-5p of lncRNAs. The StarBase website predicted the presence of complementary binding sites between Neat1 and miRNA-144-3p, Malat1 and miRNA-15b-5p (**Figure. 3c**). Subsequently, dual-luciferase reporter gene, RNA immunoprecipitation (RIP), and RNA pull-down assays were performed to explore whether LncRNA Neat1 and Malat1 acts as ceRNA to alter the miRNA-144-3p and miRNA-15b-5p expression, respectively. The results obtained from the dual-luciferase reporter gene assay exhibited that luciferase activity of LncRNA Neat1-WT and Malat1-WT were inhibited in the presence of miRNA-144-3p and miRNA-15b-5p mimic, while no evident differences was observed in corresponding mutation (**Figure. 3d**). There was a remarkably higher level of miRNA-144-3p and LncRNA Neat1, as well as miRNA-15b-5p and LncRNA Malat1 enrichment in AGO2 antibody-precipitated complex compared to the IgG antibody (**Figure. 3e**). Furthermore, RNA pull-down assay demonstrated that there were a large number of miRNA-144-3p in Neat1 and miRNA-15b-5p in Malat1 pull-down pellets (**Figure. 3f**), suggesting that miRNA-144-3p and miRNA-15b-5p had sequence specificity for LncRNA Neat1 and Malat1 recognition, respectively. Meanwhile, miRNA-144-3p and miRNA-15b-5p expression were found to be significantly decreased, while *Gad-67*, *VGAT* and *GAT-3* mRNAs increased in neuro-2a cells with LncRNA Neat1 or Malat1 over-expression (**Figure. 3g**). The aforesaid data suggested that LncRNA Neat1 and Malat1 positively regulated GABA associated genes expression and this was achieved through competitively bind to miRNA-144-3p and miRNA-15b-5p.

Additionally, the levels of Neat1 and Malat1 expression were remarkably enhanced in mPFC tissues of LBRD standard decoction administration in contrast to model samples (**Figure. 3h**). Spearman's correlation analysis showed an inverse relationship between LncRNA Neat1 and miRNA-144-3p, also in LncRNA Malat1 and miRNA-15b-5p. Whereas, there was positive association between LncRNA Neat1, Malat1 and genes related to GABA release and uptake (**Figure. 3i-j**). To conclude, LBRD standard decoction restrained the develop of depression possibly through modulating lncRNA Neat1 and Malat1 by which elevated GABA by competitively binding to miRNA-144-3p and miRNA-15b-5p.

3.5 Effects of LBRD standard decoction administration on the expression of miRNAs targeting to GABA related gene in SST-positive neurons

Finally, we examined the level of miRNA-144-3p and miRNA-15b-5p expression in SST interneuron using RNA-FISH. The results demonstrated the presence of Cy3-labeled miRNA-144-3p and Cy5-labled miRNA-15p-5p in the cytoplasm of neurons. Compared to control group, the number of miRNA-144-3p and miRNA-15p-5p positive cells was significantly decreased in mPFC tissues of depressive mice (**Figure. 4a**). Furthermore, chronic stress resulted in an obvious reduction of SST interneuron numbers in the mPFC tissues, but markedly enhanced the miRNA-144-3p level in SST-positive neurons (**Figure. 4b**). Regrettably, there was no statistical difference of miRNA-15b-5p expression in SST interneuron. While LBRD standard decoction treatments could reverse these changes (**Figure. 4a-c**). Altogether, these findings indicated that LBRD standard decoction restored the GABA secretion function of the vulnerable SST interneuron in depression by regulating the miRNA-144-3p mediated GABA synthesis, and release.

4. Discussion

TCM, with a reliable amount of clinical practice and unique curative effect in depression treatment, is gaining increasing attention [41]. LBRD standard decoction, which is a classical TCM formula with consisting of lily bulb and fresh rehmannia juice, is extensively implicated in treatment of affective disorder [8, 9, 42]. Each single herb of LBRD standard decoction is beneficial for central nervous system disorders and widely used in an Asian-medicated diet. Although the results show that LBRD is a safe and effective treatment for depression in clinical trials, it is still not clear the effective constituents, and what the underlying possible mechanisms are during the intervention and how it plays a role in this process.

In this work, there are 32 chemical constituents in LBRD standard decoction including catalpol, vidarabine, aminoadipic acid, myristic acid, verbascoside and etc. Among them, the contents of verbascoside and catalpol in LBRD standard decoction met with the standard qualities of lily bulb and raw rehmannia root in the *Pharmacopoeia of People's Republic of China* (2020 Edition). Furthermore, both of them have been reported to exert antidepressant, antioxidative, anti-inflammatory, immunoregulatory and neuroprotective effect. Thus, verbascoside and catalpol may be served as quality control marker and effective ingredients of LBRD standard decoction, which could benefit the modernization, standardization and internationalization of LBRD decoction as a compound drug.

Our previous reports demonstrated that CUMS for 4 weeks in mice could produce obvious depression-like behavior [39]. Thus, we established a 4-week CUMS mice model to observe the antidepressant effect and possible mechanism of LBRD standard decoction in CUMS-induced depression-like behavior mice. Behavioral results showed LBRD standard decoction could produce antidepressant like effect and its effect was similarly to fluoxetine, which was commonly used for depression treatment in clinic. In addition to anti-depressive effect, LBRD standard decoction was also found to effectively improve the

severity of anxiety symptoms. Meanwhile, our current results revealed the protection of LBRD standard decoction on cortex neurons may be related to reduction of glutamate and recover the balance of pro/anti-inflammatory cytokines (Figure. 1). Evidences have showed that the abnormalities in the synthesis, metabolism, and reuptake into neurons of glutamate and inflammatory factors in the brains was contributed to the pathophysiology of depression [12, 43–45]. Further preclinical and animal studies also demonstrated that acute and chronic stress procedures lead to the increased extracellular glutamate in the cortex, and this has led to glutamate-mediated exitotoxicity via actions at extra synaptic N-methyl-D-aspartate receptors (NMDARs) or GluR2-Parkin pathway mediated mitophagy, which are responsible for the loss of neurons in these brain regions [46–48].

GABAergic interneuron have a critical role in orchestrating the balance of excitation and inhibition within the mPFC tissue, as well as excitatory outputs to projection areas by targeting specific subcellular domains of glutamatergic pyramidal neurons, and controlling cortical firing rate, bursting, timing, synchrony and rhythms [49]. In current study, LBRD standard decoction intervention can achieve the balance between the Glu and GABA in mFPC tissues by increasing *Gad-67*, *VGAT* and *GAT-3* genes expression which are responsible for GABA synthesis, release and uptake. From the treatment perspective, our findings supported the latest hypothesis of depression whereby deficient GABAergic inhibition of the cortical glutamatergic system results in its over-activity [50]. Elevated Glu in depression may result from GABAergic deficit (lower GABA levels or GABAergic interneuron numbers) resulting in glutamatergic disinhibition [12]. Moreover, NMDAR antagonists including ketamine and D-cycloserine (DCS), and GABA agonists targeting Glu and GABA homeostasis have antidepressant benefit in both MDD and bipolar depression [51, 52].

GABAergic interneurons are now recognized to be highly heterogeneous in terms of multiple morphological, electrophysiological, and molecular properties. Studies have also begun to investigate the role of three major largely non-overlapping GABA interneuron subtypes (SST, PV and VIP) in the pathophysiology and treatment of depression [35]. However, there is less evidence of consistent alterations of specific GABAergic cell-type markers [37]. The reason for these discrepancies is unclear, but owing to the length of stress exposure, the type of stressor, and the method of analysis of interneuron markers. Fortunately, the consensus generating from these cellular and molecular studies reveal SST interneuron is a preferentially vulnerable GABAergic subtype to biological adversity [12, 53]. Similarly, our results revealed the number of SST interneuron and the level of SST mRNA and protein expression were decreased in stress induced depression. Reduced SST interneuron cell function alters information processing by cortical microcircuits in result of reduction of temporal and spatial signal to noise ratios of excitatory input onto pyramidal neurons [54]. Furthermore, LBRD standard decoction medications can modulate the balance of GABAergic interneuron subpopulations and reverse SST interneurons deficit in depression by improving GABA related genes expression within it (Figure. 2). Our results revealed deficits in SST interneuron represent a contributing cellular pathology, and therefore becoming a novel target for LBRD standard decoction therapeutic approaches by normalizing altered inhibitory function in depression with reduced SST cell and GABA functions.

Epigenetic modifications, i.e., DNA methylation, post-translational histone modification and interference of microRNA (miRNA) or long non-coding RNA (lncRNA), are able to influence the severity of the depression and the outcome of the therapy [55]. In this study, we successfully constructed a GABA-related ceRNA regulatory network that LncRNA Neat1 and Malat1 acts as a miRNA-144-3p and miRNA-15b-5p sponge to regulate GABA synthesis, release and uptake by mediating Gad-67, VGAT and GAT-3 (Figure. 3). LBRD standard decoction administration can remarkably enhance the levels of Neat1 and Malat1 expression and decrease the miRNA-144-3p level in SST-positive neurons (Figure. 4). LncRNA NEAT1 performs a scaffolding function in the nucleus that releases regulatory proteins after nerve cell activation, fine-tuning the excitatory response that is important for pathological seizure states. In addition, a decrease in lncRNA NEAT1 leads to altered expression of multiple gene transcripts involved in ion channel function following neuronal activation [56–58]. Notably, another lncRNA, MALAT1, which displays considerable expression in neurons, can be linked thanks to rodent models of psychiatry disease to the set of molecular alterations contributing to disease onset [59, 60]. miRNA-144-3p targets a number of genes implicated in the response to stress, aging diseases and mood stabilizer treatment including *Pten*, *Spred1*, *EGFR*, *Nrf2*, *AQP*1, *NGF*, *Brg1* and *Notch1* [61–64]. Unfortunately, there are few reports on the lncRNA Neat1 and malat1 directed miRNAs involved in the development and treatment of depression. To our knowledge this is the first time to elaborate dysfunctional lncRNA-directed regulatory network contributes to GABA deficits in depression and LBRD standard decoction can reverse reduced SST neuron activity by regulating the miRNA-144-3p mediated GABA synthesis, release (Figure. 5).

Our study has some limitations. First, LBRD standard decoction contains multiple ingredients; we have only demonstrated its antidepressant effects. The activity and synergies between the components are not yet clear and further research is needed to elucidate one or more components responsible for these effects. Second, the results of our study suggest that deregulated ceRNA network contributes to GABA deficits in SST interneuron, but how this alteration influences other GABAergic neurons subtype information processing in neuronal cortical microcircuit, has not been addressed. More importantly, whether changing the GABA-related miRNA network in SST neurons reverses the therapeutic effect of LBRD standard decoction. Therefore, in future work, we will adopt small RNA interference technology, optogenetic technology, as well as single cell sequencing to study the antidepressant effects of LBRD standard decoction based on neuroepigenetics mechanism of GABA deficits.

5. Conclusion

Overall, the current study proved that SST interneuron may be served as a preferentially vulnerable GABAergic neuronal subtype in depression and LBRD standard decoction antidepressant activities potentially relate to fight reduced SST cells activity by regulating the miRNA-144-3p mediated GABA synthesis and release. This research will offer references for gaining insight into mechanisms underlying psychopathology, and for novel antidepressant agents contained TCM formula development in depression and other brain disorders characterized by low SST cell function.

Abbreviations

ceRNA, competitive endogenous RNA; CUMS: chronic unpredictable mild stress; ELISA, enzyme linked immunosorbent assay; FST, forced swimming test; GABA transporter; GAT-3, GABA transporter-3; Gad-67, glutamate decarboxylase-1; GABA, γ-aminobutyric acid; Glu, glutamate; H&E, hematoxylin-eosin; HRP, horseradish peroxidase; IL-1β, interleukin-1β; IP, Immunoprecipitation; MDD, Major depressive disorder; mPFC, medial prefrontal cortex; MAOIs monoamine oxidases inhibitors; lncRNAs, long noncoding RNAs; NMDARS, N-methyl-D-aspartate receptors. PV, parvalbumin; SPT, preference test; SSRIs, selective serotonin reuptake inhibitor; SST, somatostatin, TCM, traditional Chinese medicine; TST, tail suspension test; UHPLC-Q-TOF/MS, ultra-high performance liquid chromatography quadrupole time of flight tandem mass spectrometry; VIP, vasoactive intestinal peptide.

Declarations

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Authors contributions: YX Xue, HX Zhang and J Pan contributed to the experiments and data analyses. QC Mao, X Chang and YT Lu contributed to draw diagram. XB Song, SJ Wang and K Ma contributed to the project design and paper writing. All authors have read and approved the final version manuscript.

Ethics declarations

Ethics approval and consent to participate: All animal experiments were approved by Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine (SDUTCM201805311223).

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Figures

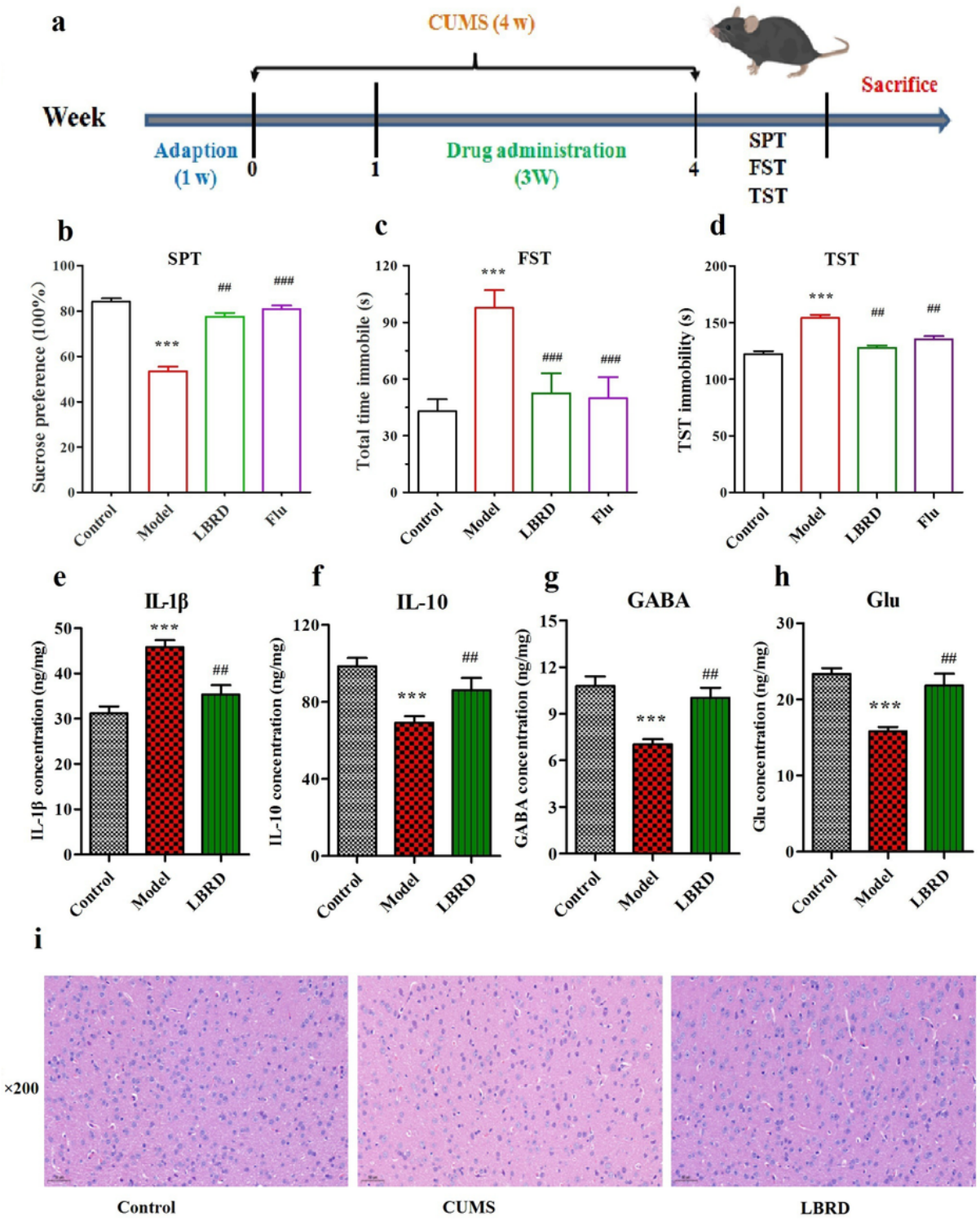

Figure 1

LBRD standard decoction ameliorated depressive-like behaviors by correcting decreased GABA and recovering the balance of pro/anti-inflammatory cytokine. (a) Schematic diagram of CUMS, drug treatment, and behavioral testing program schedule. (b) The percent of sucrose consumption/total water in the SPT. (c) Total time spent immobile in FST. (d) Total time spent immobile in TST. LBRD standard decoction treatment significantly decreased IL-1β (e), Glu (h) and increased the level of IL-10 (f) and GABA (g). (i) Effect of LBRD standard decoction on mPFC morphologies by H&E staining (×200 magnification, scale bar=50 μm). Data are expressed as mean ± SEM (n=9-12 per/group. *P<0.01, **P<0.01, ***P<0.001, compared to the control group; #P<0.05, ##P<0.01, ###P<0.001, compared to the model group. Model: CUMS+Saline group, Flu: CUMS+fluoxetine group, LBRD: CUMS+LBRD standard decoction.

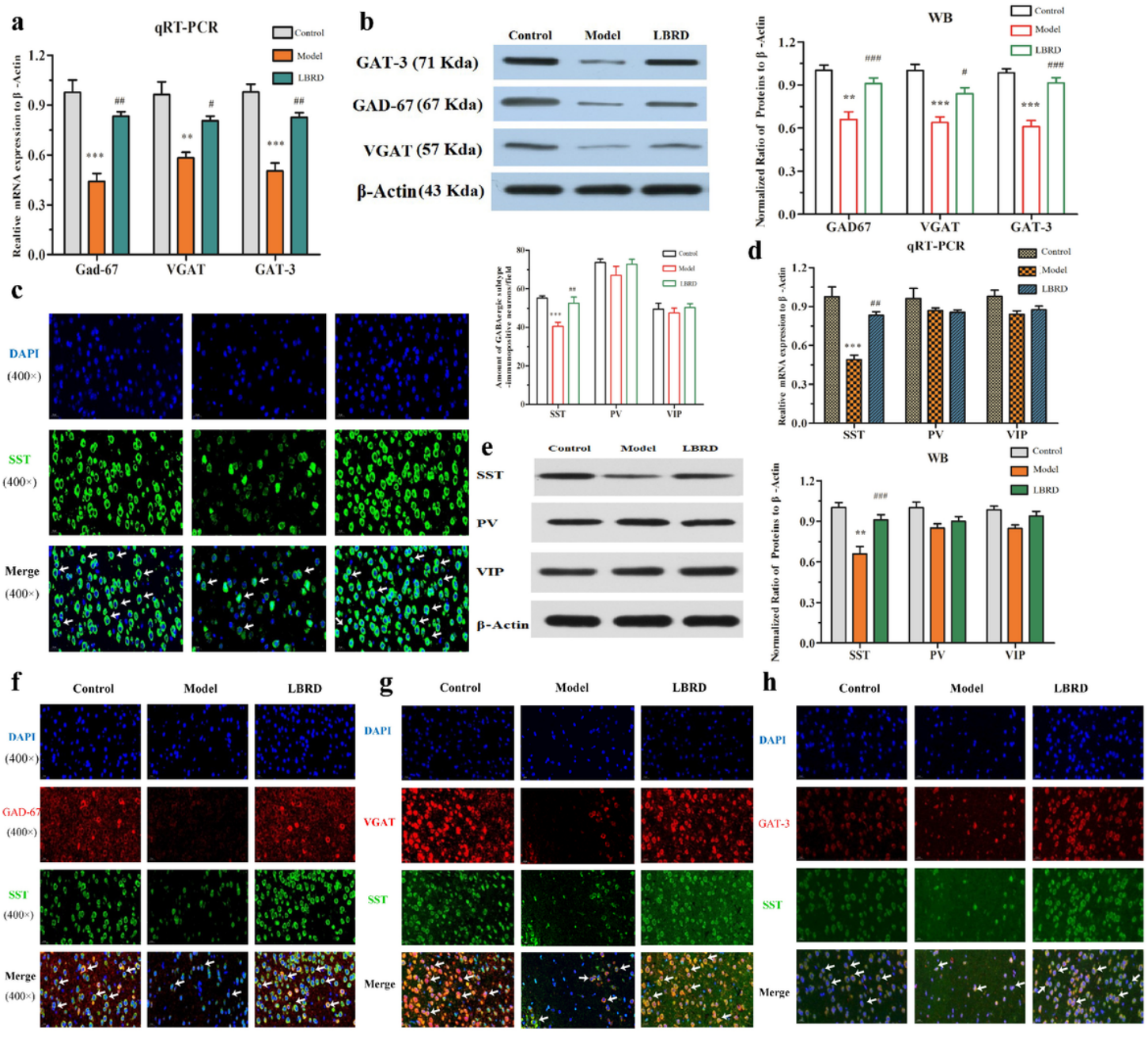

Figure 2

Effects of LBRD standard decoction administration on the GABA associated genes expression in different GABAergic interneuron subtypes. (a) qRT-PCR analysis and (b) western blot of GABA associated genes Gad-67, VGAT and GAT-3 expression level. β-actin was set as the internal control. (c) Immunofluorescence staining of mPFC tissues sections. Arrows indicate the cells positive for SST interneuron. (d) qRT-PCR analysis of co-expressed neurochemical markers of GABAergic interneuron. (e) Western blot analysis of co-expressed neurochemical markers of GABAergic interneuron (SST, PV and VIP) proteins expression level. Immunofluorescence staining of Gad-67 (f), VGAT (g) and GAT-3 (i) in the SST interneuron. DIPA (blue), SST (green), Gad-67 (red), VGAT (red) and GAT-3 (red); ×400 magnification, scale bar=20 μm; White arrows indicate the GABA associated genes expression in SST interneuron. Three non-overlapping fields of view were randomly selected in each slice under the fluorescence microscope (n=24 slices/6-8 mice/group). Data are expressed as mean ± SEM. *P<0.01, **P<0.01, ***P<0.001, compared to the control group; #P<0.05, ##P<0.01, ###P<0.001, compared to the model group. Model: CUMS+Saline group, LBRD: CUMS+LBRD standard decoction.

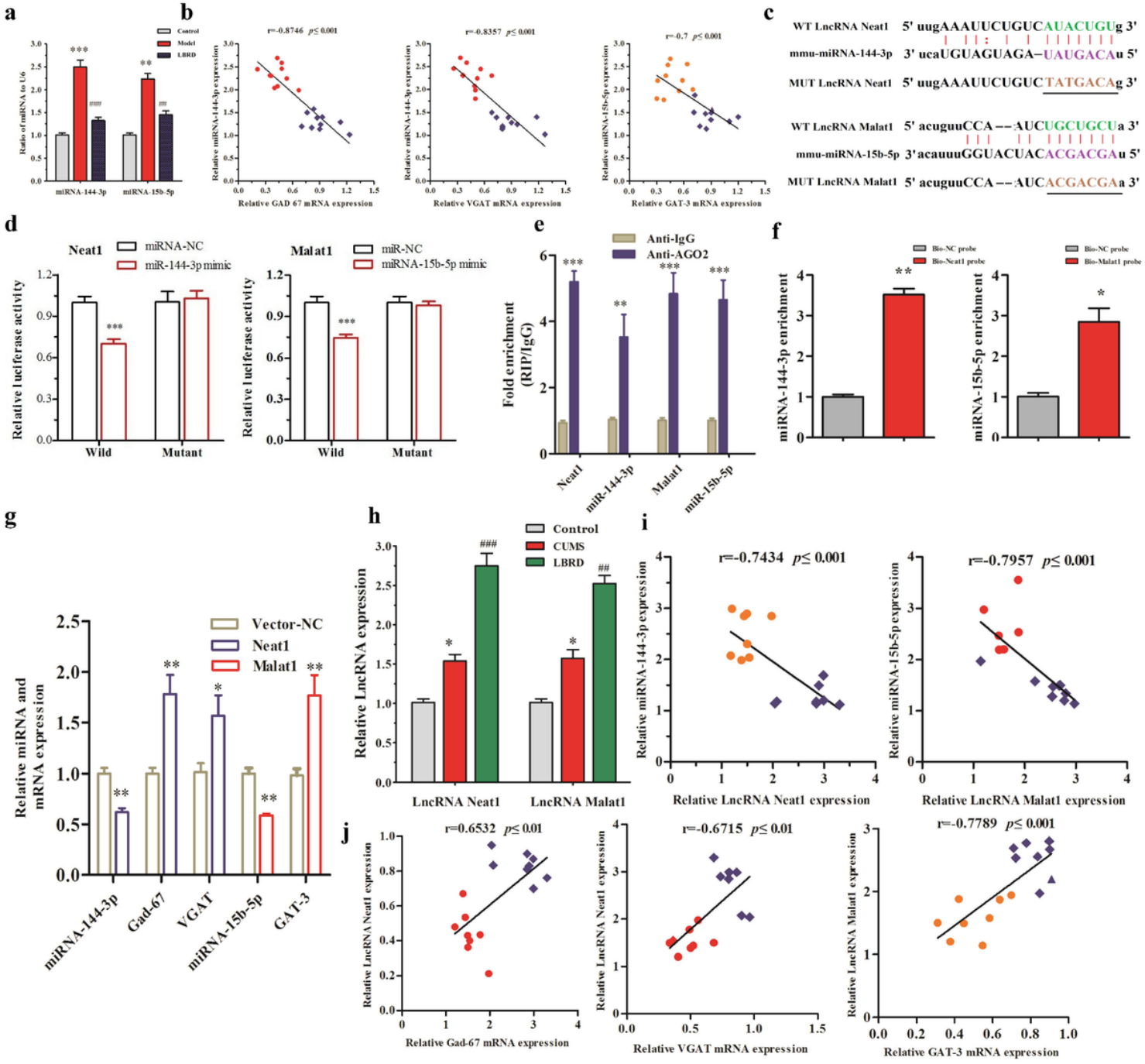

Figure 3

LncRNA Neat1 and Malat1 elevated GABA synthesis, release and uptake associated genes expression by competitively binding to miRNA-144-3p or miRNA-15b-5p. (a) qRT-PCR analysis of miRNA-144-3p and miR-15b-5p expression in mPFC tissues in mice exposed to chronic mild stress and received treatment of LBRD standard decoction. U6 was set as the endogenous control. (b) Spearman's correlation analysis was used to reveal the negative correlation between GABA associated miRNA and corresponding target genes in mFPC tissues. Blue dots indicate congregation of the LBRD standard decoction treated-group and red dots indicate congregation of the depression group (n=8 pre/group). (c) The complementary binding sites between lncRNA Neat1 and miRNA-144-3p, as well as lncRNA Malat1 and miRNA-15b-5p in

the StarBase website. (d) Luciferase activity of Neat1-WT/MUT and Malat1-WT/MUT detected by dual-luciferase reporter gene assay. (e) Enrichment of Neat1 and miRNA-144-3p, as well as Malat1 and miRNA-15b-5p by Anti-Ago2 or Anti-IgG detected by RIP assay. (f) Enrichment of miRNA-144-3p and miRNA-15b-5p detected by RNA pull-down assay. (g) The level of miRNAs and mRNAs associated with GABA release and uptake expression in neuro-2a cells with over-expressed LncRNA Neat1 and Malat1 determined by qRT-PCR. (h) qRT-PCR analysis of LncRNA Neat1 and Malat1 expression in mPFC tissues from mice with received chronic mild stress and treatment of LBRD standard decoction. (i) The relationship between LncRNA and its corresponding target prediction was assessed by Pearson's correlation coefficients. (j) The correlation between LncRNA Neat1 and Malat1 and gene related to GABA synthesis, release and uptake. Blue dots indicate congregation of the LBRD standard decoction treated-group and red dots indicate congregation of the depression group (n=8 pre/group). *P<0.01, **P<0.01, ***P<0.001, compared to the control group; #P<0.05, ##P<0.01, ###P<0.001, compared to the model group. Model: CUMS+Saline group, LBRD: CUMS+LBRD standard decoction.

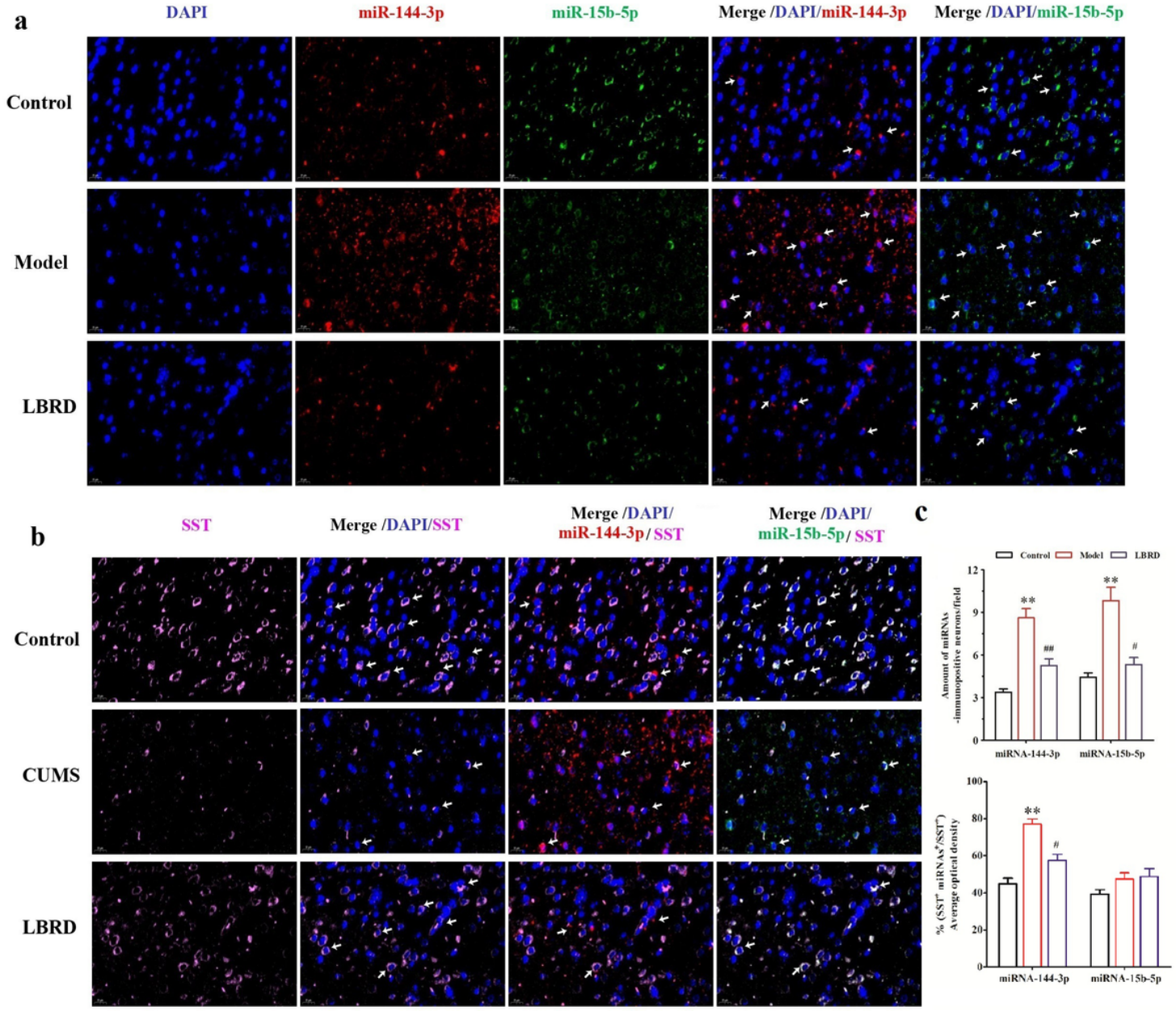

Figure 4

Effects of LBRD standard decoction administration on the expression of miRNA-144-3p and miRNA-15b-5p in SST-positive neurons. (a) miRNA-144-3p and miRNA-15b-5p localization in mFPC tissues was examined by FISH (400×). DAPI-stained nuclei were blue, Cy3-labeled miRNA-144-3p were red and Cy5-labled miRNA-15p-5p and were green. (b) The level of miRNAs expression in SST positive neurons determined by RNA-FISH, wherein SST was labeled as pink and DAPI-stained nucleus was blue (400×). (c) Statistical analysis of the integral optical density using the Image-Pro Plus 6.0 system. Three non-overlapping fields of view were randomly selected in each slice under the fluorescence microscope (n=24 slices/6-8 mice/group). White arrows indicate the positive cells. Data are expressed as mean ± SEM. **P<0.01, ***P<0.001, compared to control group; #P<0.05, ##P<0.01, compared to model group. Model: CUMS+Saline group, LBRD: CUMS+LBRD standard decoction.

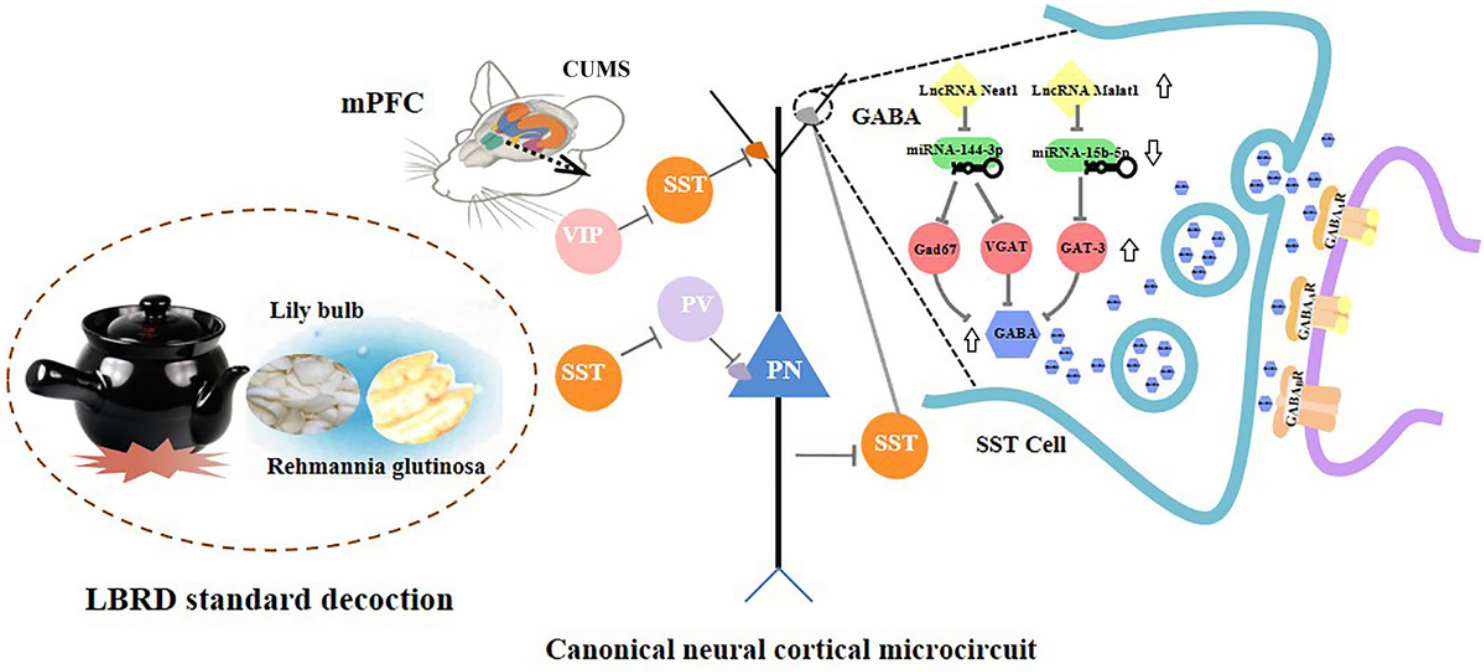

Figure 5

Schematic diagram showing the LBRD standard decoction antidepressant effects can reverse reduced SST neuron function by regulating GABA associated ceRNA network. Glutamatergic pyramidal neurons (PN) propagate excitatory signals in cortical layers and are under inhibitory control of several subtypes in GABAergic inhibitory neurons expressing various co-markers SST, PV or VIP. SST cell activity is controlled by VIP cells. SST cells are responsible for the gating and integration of excitatory inputs by targeting the apical dendrites of the PN. PV cells target the PN perisomatic region and their synchronized activity controls the propagation of action potentials over ensembles of PNs. Chronic stress leads to decreased GABA release from SST neuron. Reduced SST neuron activity alters information processing by cortical microcircuits. Thus, SST interneuron as a GABAergic neuronal subtype was preferentially vulnerable to stress induced depression. After LBRD standard decoction treatment, the GABA secretion function of the vulnerable SST cells was restored by regulating GABA associated ceRNA network.

Supplementary Files

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