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Short Communication

Inhibitory effect of yokukansan on the decrease in the hippocampal excitatory amino acid transporter EAAT2 in stress-maladaptive mice

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ABSTRACT

Chronic stress is widely recognized as a risk factor for the development of major depression and anxiety disorders. Recently, we reported that yokukansan (YKS), a traditional Japanese herbal medicine, alleviated emotional abnormality in stress-maladaptive mice. The aim of the present study was to examine the effect of YKS on the expression of excitatory amino acid transporter (EAAT) 1–4 in the prefrontal cortex and hippocampus in stress-maladaptive mice. Mice were chronically exposed to inadaptable stress, i.e. repeated restraint stress for 240 min/day for 14 days. After the final exposure to stress, brains of mice were rapidly removed and the hippocampus and prefrontal cortex were dissected. Expressions of EAAT1-4 and glial fibrillary acidic protein (GFAP), a marker of astrocytes, in the brain tissues were analyzed by western blotting. Western blot analysis revealed that the expression level of EAAT2 was specifically decreased in the hippocampus of stress-maladaptive mice while there were no changes in the level of GFAP, and this change was inhibited by chronic treatment with YKS. In contrast, no changes were observed in the levels of EAAT1, EAAT3 or EAAT4 in stress-maladaptive mice. These results suggest that YKS may protect against the decrease in hippocampal EAAT2 expression induced by stress maladaptation, and this may contribute, at least in part, to the improvement of emotional abnormality.

1. Introduction

Stress is thought to be a risk factor for psychiatric disorders, such as major depression.[1] Based on the results of preclinical studies, it is well-known that chronic stress paradigms in animals recapitulate many of the core behavioral characteristics of clinical depression.[2] Recently, we reported that mice exposed to repeated excessive restraint stress showed emotional abnormality,[3] which suggests an inability to adapt to stressful conditions. Moreover, this emotional abnormality induced by stress maladaptation was alleviated by chronic treatment with yokukansan (YKS),[3] a traditional Japanese herbal medicine that is composed of seven kinds of dried medicinal herbs, i.e., Atractylodis lanceae Rhizoma, Poria, Cnidii Rhizoma, Uncariae Uncis cum Ramulus, Angelicae Radix, Bupleuri Radix and Glycyrrhizae Radix. However, the mechanisms of this effect of YKS are still unknown.

A growing body of evidence suggests that brain glutamatergic neurotransmission may be involved in the pathogenesis of stress-related psychiatric disorders including major depression.[4–7] In the glutamatergic system, excitatory amino acid transporters (EAATs) control the concentration of glutamate in the synaptic cleft by regulating its reuptake. Several EAAT subtypes have been identified to date: EAAT1 and the most abundant EAAT2 are present in glial cells, while EAAT3 and EAAT4 are present predominantly in neurons.[8] Among them, more attention has been paid to the role of EAAT2, which is also called GLT-1, in the pathophysiology and treatment of depression. For example, a reduction of EAAT2 has

Abbreviations: EAAT, excitatory amino acid transporter; GFAP, glial fibrillary acidic protein; YKS, yokukansan.

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been reported in human postmortem studies in depressive patients,[9] and this finding is supported by a preclinical study which found that chronic restraint stress induced a significant decrease in the EAAT2 protein level in the periaqueductal gray matter.[10] More recent studies have demonstrated that animals exposed to chronic unpredictable stress showed depressive-like behaviors as well as a decrease in EAAT2 expression in the hippocampus, which are both reversed by chronic treatment with antidepressant.[11,12] These reports suggest that EAAT2 may be an attractive candidate molecule associated with stress-related emotional abnormality.

Dietary thiamine-deficient rodents have been reported to show anxiety and depressive-related behaviors.[13,14] Interestingly, recent *in vitro* studies revealed that YKS ameliorated the decrease in the mRNA expression of EAAT2 under thiamine-deficient conditions in cultured astrocytes.[15] Based on previous reports, we assume that YKS may inhibit the decrease in the expression of EAAT2 induced by chronic exposure to stress stimuli. However, sufficient *in vivo* experiments to confirm this hypothesis have not yet been performed. In the present study, we examined the effect of YKS on the expression of EAATs in the prefrontal cortex and hippocampus in stress-maladaptive mice that were repeatedly exposed to excessive restraint stress.

2. Materials and methods

2.1. Animals

Male ICR mice (Japan SLC Inc., Shizuoka, Japan) weighing 25–30 g were housed at a room temperature of 23 ± 1 °C with a 12-h light–dark cycle (light on 7:00 a.m. to 7:00 p.m.). Food and water were available ad libitum. All experiments with animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Committee on the Care and Use of Laboratory Animals of the International University of Health and Welfare.

2.2. Materials

Yokukansan is composed of seven dried medicinal herbs: 4.0 g of Atractylodis Lanceae Rhizome (rhizome of Atractylodes lancea De Candolle), 4.0 g of Poria sclerotium (sclerotium of Poria cocos Wolf), 3.0 g of Cnidium Rhizome (rhizome of Cnidium officinale Makino), 3.0 g of Uncaria Hook (hook of Uncaria rhynchophilla Miquel), 3.0 g of Japanese Angelica Root (root of Angelica acutiloba Kitagawa), 2.0 g of Bupleurum Root (root of Bulpleurum falcatum Linne') and 1.5 g of Glycyrrhiza (root and stolon of Glycyrrhiza uralensis Fisher). These herbs are registered in the Pharmacopeia of Japan ver. 17. The powdered water extract of yokukansan used in the present study was manufactured according to the formulation previously reported[16] and supplied by Tsumura & Co. (Tokyo, Japan). The manufacturing processes and quality are standardized based on the Good Manufacturing Practices defined by the Ministry of Health, Labor and Welfare of Japan. The three-dimensional high-performance liquid chromatography (3D-HPLC) profile of representative batches of yokukansan has been reported previously.[3] Yokukansan was dissolved in purified water and administered orally (p.o.) in a volume of 10 ml/kg. The dosage of YKS (1000 mg/kg) was chosen based on previous reports.[3]

2.3. Exposure to chronic restraint stress and YKS treatment

Mice were exposed to chronic restraint stress under conditions in which they could not adapt to stress, as previously described.[3] Briefly, mice were either exposed to repeated restraint stress for 240 min/day by being inserted into a syringe (50 ml) (stressed group) or left in their home cage (non-stressed group) for 14 days. From the 3rd day of exposure to stress, mice were given YKS or vehicle immediately after the daily exposure to restraint stress.

2.4. Western blotting

After the final exposure to restraint stress, mice were sacrificed by decapitation. Their brains were rapidly removed and the hippocampus and prefrontal cortex were dissected on ice. The brain tissues were homogenized in lysis buffer containing 137 mM NaCl, 0.02 mM Tris-HCl (pH 8.0), 1 % NonidetP-40, 10 % Glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/mL aprotinin, 1 mg/mL leupeptin, 0.5 mM Sodium vanadate, and 1 mM ethylenediaminetetraacetic acid, and then centrifuged to obtain the supernatants. Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Protein extracts were loaded on SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride paper (Millipore, Billerica, MA, USA). The membranes were blocked in blocking buffer containing 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.05 % Tween20, and 5 % skim milk for 1 h at room temperature and incubated with anti-EAAT1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA; diluted 1:500), anti-EAAT2 antibody (Cell Signaling, Danvers, MA, USA; diluted 1:1000), anti-EAAT3 antibody (Santa Cruz Biotechnology; diluted 1:1000), anti-EAAT4 antibody (Santa Cruz Biotechnology; diluted 1:1000) or anti-glial fibrillary acidic protein (GFAP) antibody (Millipore, diluted 1:5000) overnight at 4 °C. The membranes were washed repeatedly in Tris-buffered saline (20 mM Tris–HCl (pH 7.6), 137 mM NaCl) containing 0.05 % Tween-20, and then horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA; diluted 1:10000) was added for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnology), and scanned, optimized and analyzed by Chemi Doc XRS (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative results were expressed as the ratio of the band intensity of the protein of interest to the band intensity of GAPDH.

2.5. Statistical analysis

The data are presented as the mean ± S.E. Statistical significance was assessed by one-way analysis of variance followed by post hoc Tukey's multiple comparison tests. p values of <0.05 were considered statistically significant.

3. Results

3.1. Effect of YKS on EAAT expression in the prefrontal cortex and hippocampus of stress-maladaptive mice

Western blotting revealed that the levels of EAAT1 expression in the prefrontal cortex and hippocampus were not affected by chronic exposure to restraint stress and treatment with YKS (Fig. 1A). In contrast, a significant decrease in the expression level of EAAT2 was observed in the hippocampus, but not the prefrontal cortex, of stressed mice, and this change in EAAT2 expression was suppressed by treatment with YKS (Fig. 1B). There were no significant differences in the expression levels of EAAT3 or EAAT4 in the prefrontal cortex and hippocampus among the non-stressed, stressed and YKS plus stressed groups (Fig. 1C and D). In addition, treatment with YKS did not affect the expression levels of EAAT1, EAAT2, EAAT3 or EAAT4 in the prefrontal cortex or hippocampus of non-stressed mice (data not shown).

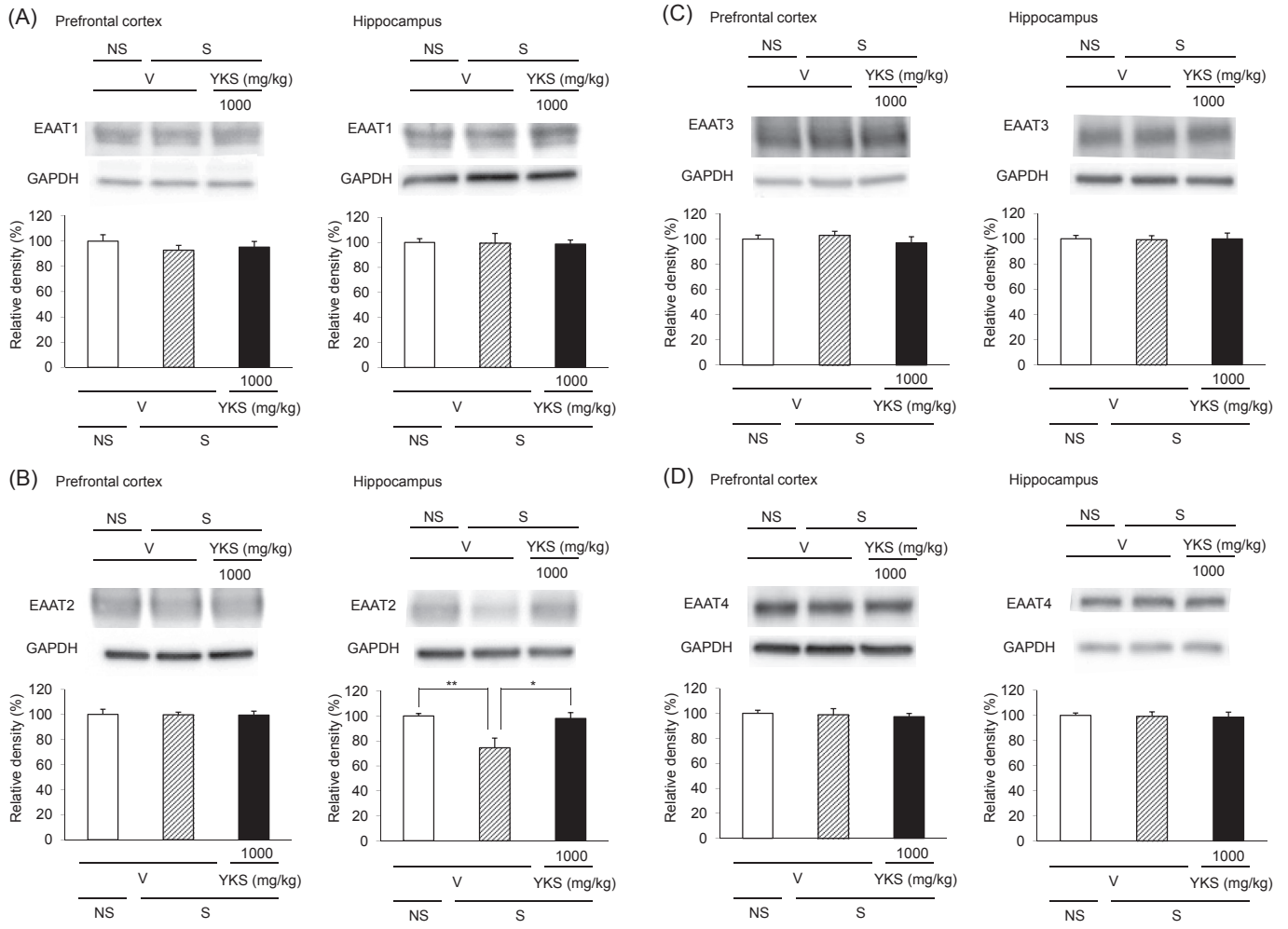

Fig. 1. Effect of YKS on EAAT expression in the prefrontal cortex and hippocampus of stress-maladaptive mice. Photographs show representative western blots of EAAT1 (A), EAAT2 (B), EAAT3 (C), and EAAT4 (D). Graphs show the relative densities of bands. Quantitative data were normalized and expressed as a percentage of the expression of EAAT1, EAAT2, EAAT3 and EAAT4 in the vehicle (V) plus non-stressed (NS) group. Each column represents the mean with SE of 8 mice. *$p < 0.05$, **$p < 0.01$.

3.2. Effect of YKS on GFAP expression in the prefrontal cortex and hippocampus of stress-maladaptive mice

To examine whether the decrease in the expression level of EAAT2 was associated with a decrease in astrocytes, we determined the amount of GFAP protein, a marker of astrocytes. Western blotting showed that the protein levels of GFAP in the hippocampus and prefrontal cortex were not affected by chronic exposure to restraint stress and treatment with YKS (Fig. 2).

4. Discussion

In the present study, we tried to characterize the expression pattern of EAAT1-4 in the prefrontal cortex and hippocampus of mice that were not able to adapt to excessive stress, and observed the changes in specific EAAT protein levels. As a result, the protein level of EAAT2 was decreased in the hippocampus of mice that had been exposed to inadaptable stress (240 min restraint stress once a day for 14 days), while there were no changes in the protein levels of other EAATs in the hippocampus and prefrontal cortex. EAAT2 is known to be the most active glutamate transporter, and regulates almost all extracellular glutamate reuptake.[17] A previous report showed that repeated immobilization stress resulted in a significant increase in extracellular glutamate levels in the hippocampus of rats.[18]

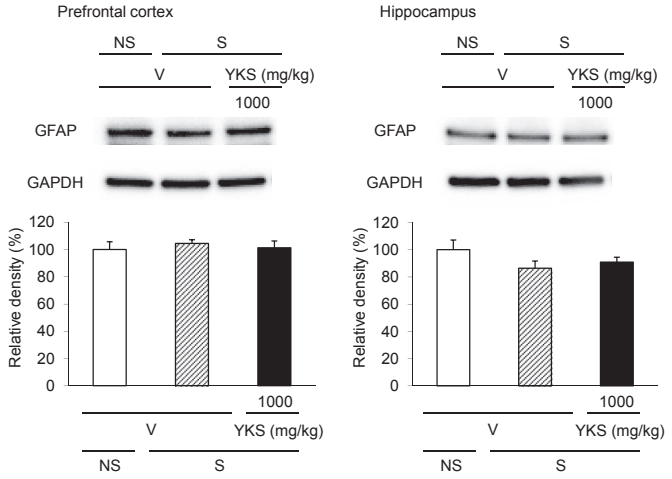

Fig. 2. Effect of YKS on GFAP expression in the prefrontal cortex and hippocampus in stress-maladaptive mice. Photographs show representative western blots of GFAP. Graphs show the relative densities of bands. Quantitative data were normalized and expressed as a percentage of the expression of GFAP in the vehicle (V) plus non-stressed (NS) group. Each column represents the mean with SE of 8 mice.

Therefore, the reduced expression of EAAT2 in the hippocampus observed in the present study may cause impaired glutamate transport with elevated extracellular glutamate levels. Furthermore, previous reports showed that the expression level of EAAT2 was reduced in the hippocampus of learned-helplessness rats, an animal model of depression,[19] and in the cerebral cortex obtained from suicidal subjects with major depressive disorder.[9] Importantly, we confirmed that the condition of chronic stress exposure used in the present study disrupts the ability to adapt to stress and induces emotional abnormality.[3] Therefore, a decrease in hippocampal EAAT2 expression, which leads to an excessive extracellular glutamate level, may be involved, at least in part, in the mechanism that underlies the disruption of the ability to adapt to emotional stress.

Another finding in the present study was that chronic treatment with YKS immediately after daily exposure to stress suppressed the decrease in the expression level of EAAT2 in the hippocampus induced by inadaptable stress. In contrast, there was no change in EAAT2 expression under chronic treatment with YKS in non-stressed mice. These findings indicate that YKS is specifically effective for restoring decreased EAAT2 expression under chronically stressful conditions. YKS has been reported to decrease the extracellular glutamate level in the ventral posterior medial thalamus of thiamine-deficient (TD) rats that were produced by feeding a TD diet.[14] Also, *in vitro* studies with cultured astrocytes have revealed that YKS ameliorated the decrease in glutamate uptake as well as the mRNA expression of EAAT2 under thiamine-deficient conditions,[15] suggesting that YKS may be able to lower the glutamate level by increasing the expression of EAAT2. To our knowledge, the present study is the first to present *in vivo* evidence to support this hypothesis. Furthermore, we previously found that, under the same conditions as in the present study, YKS inhibits the emotional abnormality observed in stress-maladaptive mice.[3] Taken together, these previous and present findings suggest that YKS may have a beneficial effect on emotional stress adaptation by restoring hippocampal EAAT2 expression and decreasing the extracellular glutamate level.

It is well known that EAAT2 is most abundantly present in astrocytes.[20] The present study confirmed that the protein levels of GFAP, a marker of astrocytes, in the hippocampus and prefrontal cortex were not affected by chronic exposure to stress or by treatment with YKS. This suggests that the changes in the expression level of EAAT2 observed in the present study may not be due to the atrophy of astrocytes. Although the detailed mechanisms are still unknown, it has been reported that the activation of PKC with phorbol ester caused a decrease in EAAT2 cell-surface expression in both primary co-cultures of neurons and astrocytes that endogenously express EAAT2 and C6 glioma cells transfected with EAAT2.[21] Additionally, prolonged treatment with phorbol ester decreases total EAAT2 protein levels in C6 glioma cells transfected with EAAT2.[22] Recent reports have demonstrated that YKS, as well as glycyrrhizin, a main component of glycyrrhiza, and its metabolite 18β-glycyrrhetinic acid, inhibited PKC activity in a concentration-dependent manner *in vitro*.[23] Thus, we can speculate that these active substances in YKS might increase the expression of EAAT2 by inhibiting PKC activity. Further studies will be needed to clarify this hypothesis.

In conclusion, the present findings suggest that YKS may be able to restore the decreased hippocampal EAAT2 expression under inadaptable stressful situations. This may be one of the mechanisms that underlie our previous behavioral findings that YKS has a beneficial effect on the process of stress adaptation and alleviates the emotional abnormality associated with stressful situations. The present findings provide further evidence that YKS may be effective for the clinical treatment of mental illness that results from maladaptive coping with stressful situations, such as adjustment disorder.

Conflict of interests

The authors declare that they have no conflict of interests to disclose.

References

1. Breslau N, Davis GC. Chronic stress and major depression. *Arch Gen Psychiatry*. 1986;43:309–314.
2. Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology*. 2005;52:90–110.
3. Tsuji M, Takeuchi T, Miyagawa K, et al. Yokukansan, a traditional Japanese herbal medicine, alleviates the emotional abnormality induced by maladaptation to stress in mice. *Phytomedicine*. 2014;21:363–371.
4. Ferrero AJ, Cereseto M, Reinés A, et al. Chronic treatment with fluoxetine decreases seizure threshold in naïve but not in rats exposed to the learned helplessness paradigm: correlation with the hippocampal glutamate release. *Prog Neuropsychopharmacol Biol Psychiatry*. 2005;29:678–686.
5. Grahn RE, Watkins LR, Maier SF. Impaired escape performance and enhanced conditioned fear in rats following exposure to an uncontrollable stressor are mediated by glutamate and nitric oxide in the dorsal raphe nucleus. *Behav Brain Res*. 2000;112:33–41.
6. Hunter AM, Balleine BW, Minor TR. Helplessness and escape performance: glutamate-adenosine interactions in the frontal cortex. *Behav Neurosci*. 2003;117:123–135.
7. Petty F, McChesney C, Kramer G. Intracortical glutamate injection produces helpless-like behavior in the rat. *Pharmacol Biochem Behav*. 1985;22:531–533.
8. Beart PM, O'Shea RD. Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br J Pharmacol*. 2007;150:5–17.
9. Choudary PV, Molnar M, Evans SJ, et al. Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. *Proc Natl Acad Sci U. S. A*. 2005;102:15653–15658.
10. Imbe H, Kimura A, Donishi T, Kaneoke Y. Chronic restraint stress decreases glial fibrillary acidic protein and glutamate transporter in the periaqueductal gray matter. *Neuroscience*. 2012;223:209–218.
11. Chen JX, Yao LH, Xu BB, et al. Glutamate transporter 1-mediated antidepressant-like effect in a rat model of chronic unpredictable stress. *J Huazhong Univ Sci Technolog Med Sci*. 2014;34:838–844.
12. Liu WX, Wang J, Xie ZM, et al. Regulation of glutamate transporter 1 via BDNF-TrkB signaling plays a role in the anti-apoptotic and antidepressant effects of ketamine in chronic unpredictable stress model of depression. *Psychopharmacology*. 2016;233:405–415.
13. Nakagawasai O, Yamadera F, Iwasaki K, et al. Preventive effect of kami-untanto on performance in the forced swimming test in thiamine-deficient mice: relationship to functions of catecholaminergic neurons. *Behav Brain Res*. 2007;177:315–321.
14. Ikarashi Y, Iizuka S, Imamura S, et al. Effects of yokukansan, a traditional Japanese medicine, on memory disturbance and behavioral and psychological symptoms of dementia in thiamine-deficient rats. *Biol Pharm Bull*. 2009;32:1701–1709.
15. Kawakami Z, Kanno H, Ueki T, et al. Neuroprotective effects of yokukansan, a traditional Japanese medicine, on glutamate-mediated excitotoxicity in cultured cells. *Neuroscience*. 2009;159:1397–1407.
16. Mizukami K, Asada T, Kinoshita T, et al. A randomized cross-over study of a traditional Japanese medicine (kampo), yokukansan, in the treatment of the behavioural and psychological symptoms of dementia. *Int J Neuropsychopharmacol*. 2009;12:191–199.
17. Tanaka K, Watase K, Manabe T, et al. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science*. 1997;276:1699–1702.
18. Zhu MY, Wang WP, Huang J, Feng YZ, Regunathan S, Bissette G. Repeated immobilization stress alters rat hippocampal and prefrontal cortical morphology in parallel with endogenous agmatine and arginine decarboxylase levels. *Neurochem Int*. 2008;53:346–354.
19. Zink M, Vollmayr B, Gebicke-Haerter PJ, Henn FA. Reduced expression of glutamate transporters vGluT1, EAAT2 and EAAT4 in learned helpless rats, an animal model of depression. *Neuropharmacology*. 2010;58:465–473.
20. Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J Neurosci*. 1997;17:8363–8375.
21. Kalandadze A, Wu Y, Robinson MB. Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter. Requirement of a carboxyl-terminal domain and partial dependence on serine 486. *J Biol Chem*. 2002;277:45741–45750.
22. Sheldon AL, González MI, Krizman-Genda EN, Susarla BT, Robinson MB. Ubiquitination-mediated internalization and degradation of the astroglial glutamate transporter, GLT-1. *Neurochem Int*. 2008;53:296–308.
23. Kawakami Z, Ikarashi Y, Kase Y. Glycyrrhizin and its metabolite 18 beta-glycyrrhetinic acid in glycyrrhiza, a constituent herb of yokukansan, ameliorate thiamine deficiency-induced dysfunction of glutamate transport in cultured rat cortical astrocytes. *Eur J Pharmacol*. 2010;626:154–158.