Journal of Traditional and Complementary Medicine 8 (2018) 81–88

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original Article

Sansoninto, a traditional herbal medicine, ameliorates behavioral abnormalities and down-regulation of early growth response-1 expression in mice exposed to social isolation stress

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ARTICLE INFO

Article history:
Received 28 September 2016
Received in revised form
9 February 2017
Accepted 2 March 2017
Available online 12 April 2017

Keywords:
Attention-deficit/hyperactivity disorder
Social isolation mice
Sansoninto
Early growth response 1
Sociability deficit

ABSTRACT

Social isolation (SI) mice exhibit behavioral abnormalities such as impairments of sociability- and attention-like behaviors, offering an animal model of neurodevelopmental disorders such as attention-deficit/hyperactivity disorder (ADHD). This study aimed to identify the effects of Sansoninto (SST; 酸棗仁湯 suān zǎo rén tāng) on the psychiatric symptoms related to ADHD using SI mice. Four-week-old mice were socially isolated during the experimental period, and SST administration (800 or 2400 mg/kg, p.o.) was started at 2 weeks after starting SI. SST ameliorated SI-induced impairments of sociability- and attention-like behaviors in a dose-dependent manner, and tended to ameliorate contextual- and auditory-dependent fear memory deficit. Moreover, the expression level of Egr-1 was down-regulated by SI stress, and was restored by a high dose of SST. These findings suggest that SST is useful for improvement of psychiatric disorders such as ADHD.

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a childhood neurodevelopmental disorder, characterized by inattention, hyperactivity, impulsivity, etc. The symptoms of ADHD usually disappear with age; however, it is of major concern that about 65% of ADHD patients do not recover completely, even after reaching adulthood.[1] Although the pathogenesis of ADHD is still unclear, it has been suggested that it is a functional disorder of the frontal lobe, in which dopamine acts as a neurotransmitter.[2] It has been reported that ADHD develops by a combination of various genetic variations in such areas as dopamine transporters[3–5] and receptors,[6,7] serotonin 5-HT_{2A} receptors,[8] and N-methyl-D-aspartate glutamate receptor subunits.[9] Moreover, the onset of ADHD is also affected by environmental factors, suggesting epigenetic regulation in the disorder.[10]

Many animal models are used for the research of ADHD.[11–13] We have previously proposed that social isolation (SI) rearing of rodents during early weaning may offer a viable model animal because it induces some behavioral abnormalities that are similar to those in ADHD patients, such as an increased aggressive response, attention deficit-like behavior, hyperactivity, and attenuation of the pentobarbital-induced sleep duration. Moreover, we recently found that SI stress in mice causes not only impairment of sociability and spatial attention, but also cognitive deficits in fear conditioning tests.[14,15] Neurochemical studies in our laboratory also demonstrated that SI stress decreases the expression level of early growth response 1 (Egr-1), an immediate early gene, which is an important transcription factor involved in synaptic plasticity,[16] in a manner reversible by tacrine, an acetylcholinesterase inhibitor.[15] The down-regulated expression of Egr-1 has been found in postmortem prefrontal cortices of schizophrenic patients.[17] Thus, SI mice show characteristics resembling symptoms of ADHD patients and have been useful as an environment-dependent model of ADHD.

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; Egr-1, early growth response-1; GH, group-housed; SI, social isolation.

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

http://dx.doi.org/10.1016/j.jtcme.2017.03.004

Methylphenidate, a dopamine transporter inhibitor, is one of the major drugs clinically employed for patients with ADHD or depression, and is considered to ameliorate abnormal behavior in ADHD patients, partly via reversing the down-regulated function of dopaminergic systems in the frontal lobe.[18] However, since this drug generally needs to be taken long-term by the patients, it is likely to cause various side-effects, including hallucination and insomnia, because of its intrinsic psychostimulant activity. Therefore, it is important to develop therapeutic agents for ADHD that are safer and more effective than methylphenidate.

Traditional herbal medicines, including Kampo medicines and traditional Chinese medicines, have long been used to treat or relieve the symptoms of many diseases. Moreover, clinical studies subjecting psychological disorder patients to Kampo medicine treatment have shown that some traditional herbal medicines reduce the positive and negative syndrome scale for Schizophrenia,[19] as well as reduce the symptoms of inattention, hyperactivity and impulsivity in children and adolescents.[20] These studies raise the possibility that novel agents effective for psychological disorders may be found from the traditional herbal remedies. We also have reported that several traditional herbal medicines or medicinal herbs, such as *Butea superba*,[21,22] *Bacopa monnieri*[23] and Chotosan (鉤藤散gōu téng sǎn),[24] ameliorate cognitive and emotional deficits in several types of model mice. Sansoninto (SST; 酸棗仁湯 suān zǎo rén tāng) is a traditional herbal medicine that has been used in China, Taiwan, and Japan for adult patients with insomnia, depression, and neuropathy. Moreover, Saito et al. reported that SST reverses several stress-induced decreases in pentobarbital sleep of mice.[25] These medicinal efficacies of sansoninto are speculated to be due to the anxiolytic effects of Zizyphi Semen (酸棗仁 suān zǎo rén), a main ingredient of this traditional herbal medicine.[26,27] However, the underlying mechanism of SST on the amelioration of SI-induced behavioral abnormalities has not yet been reported. In this study, we examined the effects of SST on SI mice by behavioral and neurochemical analysis.

2. Materials and methods

2.1. Preparation and chemical profiling of SST

The medicinal herbs included in SST were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). SST was extracted from a mixture of 15.0 parts *Zizyphi Semen*, 4.0 parts *Poria* (茯苓 fú líng), 3.0 parts *Cnidii Rhizoma* (川芎 chuān xiōng), 3.0 parts *Anemarrhenae Rhizoma* (知母 zhī mǔ) and 1.0 part *Glycyrrhizae radix* (甘草 gān cǎo). The yield of SST extract was 12.6%.

To identify the chemical constituents of SST, LC–MS analyses were performed with a Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface. The ESI parameters were as follows: source voltage, +4.5 kV; capillary temperature, 200 °C; and nebulizer gas, 1.5 l/min. A Waters Atlantis T3 column (2.1 mm × 100 mm) was used, and the column temperature was maintained at 40 °C. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution and (B) acetonitrile under the following gradient conditions: 0–30 min; linear gradient from 10% to 100% B, and 30–40 min; isocratic at 100% B. The flow rate was 0.15 ml/min. Mass spectrometry data obtained from the extract have been listed in the MassBank database[28] and stored in the Wakan-Yaku DataBase system (http://wakankensaku.inm.u-toyama.ac.jp/wiki/LCMS:Sansoninto_INM-749), Institute of Natural Medicine, University of Toyama.

2.2. Animals

The study was conducted according to the experimental schedule described in Fig. 1. Four-week old male ICR mice were obtained from Japan SLC (Shizuoka, Japan). Animals were housed in groups of 4–5 mice/cage (24 × 17 × 12 cm) or socially isolated (SI) in the same size cage as previously reported.[15] Housing room was maintained at 24 ± 1 °C with 65% humidity and a 12-h light-dark cycle (lights on: 07:00–19:00). Food and water were given ad libitum. All animal research procedures used in this study were in accordance with the Guiding Principles for the Care and Use of Animals (NIH Publications No. 80-23, revised in 1996). This study was also approved by the Institutional Animal Use and Care Committee of the University of Toyama.

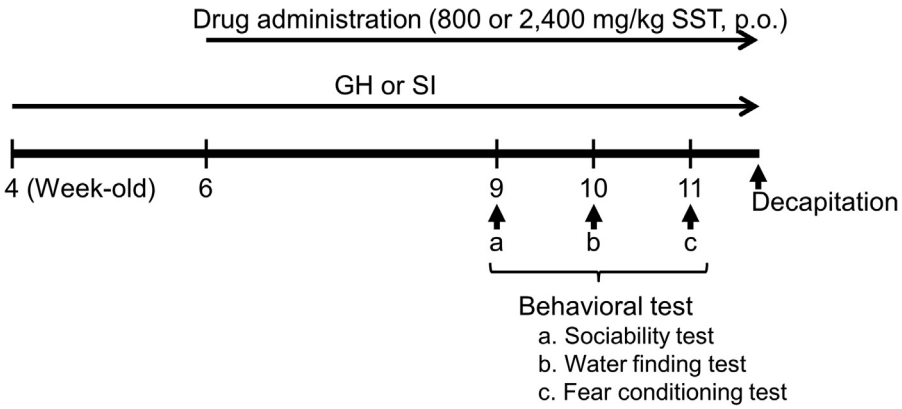

Fig. 1. Experimental schedule. Four-week-old male mice were housed in groups (GH) or socially isolated (SI) during an experimental period. The SST administration was started at 2 weeks after starting SI. The sociability test, water-finding test and fear conditioning tests were conducted at ages of 9, 10 and 11 weeks, respectively. After completing the auditory fear-conditioning test, animals were decapitated for neurochemical studies.

2.3. Drug treatment

SST administration (800 or 2400 mg/kg) at doses approximately 3 and 10 times more than the typical daily doses for human therapy was started at 2 weeks after starting SI, a period sufficient to induce behavioral abnormalities in our previous study,[14] and performed during the experimental period. SST was orally administered once daily, while GH and SI vehicle groups were given water.

2.4. Behavioral analysis

2.4.1. Sociability test

Sociability test was performed at 5 weeks after starting SI according to Okada et al.[15] The equipment was an open field

(50 cm × 50 cm × 50 cm) with black floor and gray walls. In the sociability test, a training trial was performed first. Each mouse was placed in the square arena, where two empty clear cylindrical cages (diameter 10 cm and height 12 cm) were placed diagonally, and allowed to explore the arena freely for 5 min. The time the mouse spent near (around 2 cm) the two cages was measured. The box arena and cages were cleaned using 70% ethanol between trials to prevent mice from reselecting due to olfactory cues. The test trials were performed 30 min after the training trials. An unfamiliar mouse (Stranger) was put in one of the cages, while the other cage remained empty. The total time spent near the two cages was again measured and analyzed automatically using the Smart® system (PanLab, S.L., Barcelona, Spain).

2.4.2. Water-finding test

A water-finding test was performed at 6 weeks after starting SI, according to Ouchi et al.[14] The equipment consisted of an open field (30 cm × 30 cm × 30 cm high) with a small space (10 cm × 10 cm × 10 cm) in the middle of one of the walls of the open field. A drinking nozzle was set on the center of the small space ceiling and had its end 5 and 7 cm above the floor in the training and test trials, respectively. The training and test trials were conducted on day 1 and day 2, respectively. In the training trial, each mouse was placed in one corner of the open field. The mice were allowed to explore the equipment for 5 min. Animals that could not find the drinking nozzle during the 5-min observation period were excluded from the test trials. The mice were deprived of water for 24 h after the training trial. In the test trials, the animals were again placed individually into the equipment, and the latency for drinking water (drinking latency) was measured for each animal as an index of attention-related behavior according to Ouchi et al.[14]

2.4.3. Fear-conditioning test

The fear conditioning test was performed at 6 weeks after starting SI in accordance with Okada et al., but with minor modifications.[15] Briefly, the chamber for fear conditioning consisted of a clear acrylic chamber (30 cm × 30 cm × 30 cm) and a stainless-steel grid floor equipped with an electric shock generator/scrambler SGS-002®, CS Controller CSS-001®, and Cycle Timer CMT® (Muromachi Kikai. Co. Ltd., Tokyo, Japan). The equipment was placed in a soundproof observation box (MC-050/CM, Muromachi Kikai, Co. Ltd., Tokyo, Japan), through which an auditory tone (Sonalert®, Mallory Sonalert Products Inc., Indianapolis, IN, USA) was delivered to the animal. In the training trial, animals were placed individually into the chamber and allowed to explore freely for 3 min. They then received an acoustic tone (2.9 kHz, 20 s, 80 dB) that co-terminated with electric foot shocks (0.8 mA, 2 s). The tone-foot shock pairing was repeated five times at 1-min intervals. One minute after the final foot shock delivery, the mice were returned to their home cage. Contextual and auditory fear memories were elucidated 24 h and 5 days after the training trial, respectively. In the contextual memory test, mice were placed in the same chamber to provide contextual stimuli and allowed to move freely for 6 min. One minute after placing the animal in the chamber, freezing behavior during a 5-min period was recorded as an index of contextual-dependent fear memory. For measurement of the auditory-dependent fear memory, mice were placed in the chamber for a total of 6 min. After a 3-min habituation period, the tone was delivered continuously for 3 min. The freezing behavior during the 3-min period was recorded as auditory-dependent fear memory. Animal behavior was video-recorded and analyzed automatically using the Smart® system. Freezing was defined as the absence of any movement, except for that related to respiration, and analyzed as a state with a movement speed no greater than 0.05 cm^2/s.[14,15]

2.5. Western blotting analysis of Egr-1 in cortical and hippocampus tissues

The expression of Egr-1 was analyzed using western blotting as previously described.[15] Briefly, the prefrontal cortices and hippocampi were obtained from each animal group and homogenized in 0.5 ml ice-cold buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 1.2 mg/ml aprotinin, 10 mg/ml leupeptin, pH 7.5) and centrifuged at 1500 × g at 4 °C for 3 min (Kubota 3740, Kubota Co., Tokyo, Japan). Supernatant was used in the experiments. Protein concentrations of the samples were determined by using a BCA™ protein assay kit (Thermo Scientific, Rockford, IL, USA). Aliquots of protein extracts containing 5 μg proteins were applied on SDS-polyacrylamide gels (SDS-PAGE) and electrophoresed. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immuno-Blot® membrane, Bio-Rad Laboratories, Hercules, CA, USA). Blots were blocked with 5% skim milk in 0.1% Tween 20 containing Tris-buffered saline (TBS-T) and probed with an anti-Egr-1 rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, USA) with a 1:1000 dilution or anti-β-actin mouse monoclonal antibody (Abcam, Cambridge, UK) with a 1:10000 dilution. After washing in TBS-T, the blots were incubated with anti-rabbit or anti-mouse secondary antibodies linked with horseradish peroxidase (Cell Signaling Technology, MA, USA). The chemiluminescence was detected by Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Immunoreactive bands were visualized and analyzed with ImageQuant LAS-4000 and ImageQuant TL® (GE Healthcare Japan, Tokyo, Japan).

2.6. Statistics

Data are expressed as the mean ± S.E.M. and were analyzed by unpaired or paired Student's t-test for two groups or one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. Differences of $p < 0.05$ were considered significant. The analysis was conducted using SigmaStat® ver 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

3. Results

3.1. SI stress impairs sociability and is reversed by SST

We carried out the sociability test to determine the effect of SST on SI-induced sociability deficit (Fig. 2). In the test trial, GH mice took significantly more interest in the stranger cage with the mouse ($t = 3.404$, $df = 18$, $p = 0.003$). However, there was no difference in the exploratory time for stranger and empty cages in SI mice ($t = -0.455$, $df = 18$, $p = 0.0655$), suggesting a dysfunction in social interaction behavior. In low- and high-dose SST-treated SI mice, exploratory time of the stranger cage was significantly greater than that of the empty (Low: $t = 3.630$, $df = 18$, $p = 0.002$; High: $t = 4.601$, $df = 16$, $p < 0.001$).

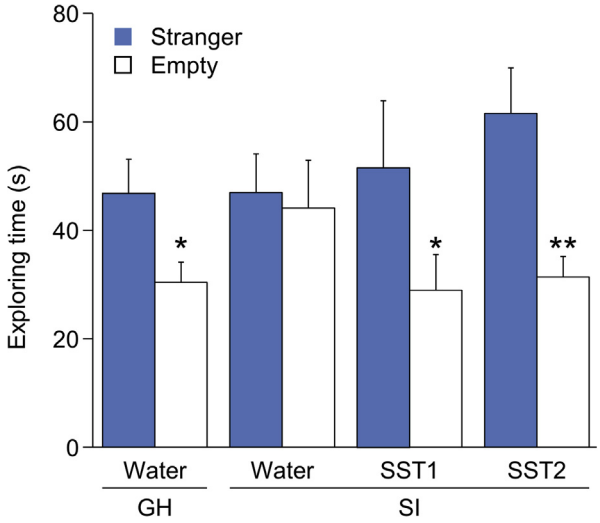

Fig. 2. The effects of SST on SI-induced sociability deficit in mice. The test was conducted at the age of 9 weeks. In the training trial, animals were allowed to freely explore the arena, in which two identical empty chambers were placed, and acclimate to the experimental environment and procedure for 5 min. In the test trial conducted 30 min after the training trial, the time a mouse spent exploring around the stranger and empty chambers was measured, as described in the text. SST was given orally at doses of 800 (SST1) and 2400 (SST2) mg/kg for 3 weeks before the test. Each data column represents mean ± S.E.M. of 11 mice. *p < 0.05, **p < 0.01 vs. the time each mouse spent around the empty chamber.

3.2. SST ameliorated SI stress-induced latent learning performance deficit in the water-finding test

The effect of SST on SI-induced spatial attention deficit was examined with a water-finding test (Fig. 3). Compared with the GH mice, SI mice had significantly increased drinking latency ($p < 0.05$). Moreover, the administration of high-dose SST (2400 mg/kg, p.o.) significantly reduced the SI-induced increase in the latency [$F(2, 31) = 3.412$, $p = 0.047$], whereas low-dose (800 mg/kg, p.o.) had no effect. These results indicate that SST ameliorates the SI-induced spatial attention deficit.

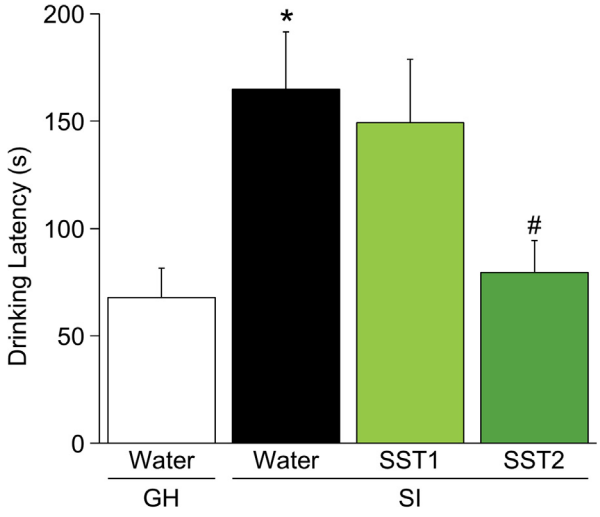

Fig. 3. The effect of SST on SI-induced attention deficit-like behavior in mice. The test was conducted at the age of 10 weeks and drinking latency of each animal was recorded as described in the text. SST was given orally at doses of 800 (SST1) and 2400 (SST2) mg/kg for 4 weeks before the test. Each data column represents the mean ± S.E.M. of 11 mice. *p < 0.05 compared with GH mice. #p < 0.05 compared with saline-administered SI group.

3.3. SST tended to ameliorate the social isolation stress-induced fear memory deficit in the fear conditioning test

We performed the fear conditioning test in order to determine the effects of SST on long-term fear memory deficit in SI mice (Fig. 4). In this test, freezing responses to contextual stimuli are dependent on the hippocampus, whereas freezing responses to auditory stimuli are dependent on the amygdala. Freezing times of SI mice from the contextual and auditory stimuli were significantly lower than in GH mice (contextual: $t = 2.583$, $df = 22$, $p = 0.017$; auditory: $t = 2.495$, $df = 21$, $p = 0.021$). On the other hand, SST slightly increased the freezing time, but not significantly.

3.4. SST reversed the SI-induced down-regulation of Egr-1 expression in the brain

After the behavioral analysis, we confirmed the effect of SI on Egr-1 expression levels in the hippocampus and frontal cortex sections. As shown in Fig. 5, SI for 7 weeks significantly reduced the expression levels of Egr-1 in the hippocampus and frontal cortex (the frontal cortex: $t = 3.963$, $df = 6$, $p = 0.007$; the hippocampus: $t = 2.479$, $df = 6$, $p = 0.048$). The administration of high-dose of SST significantly restored the expression levels of Egr-1 in the brain [the frontal cortex: $F(2,11) = 9.767$, $p = 0.006$; the hippocampus: $F(2,11) = 4.552$, $p = 0.043$].

4. Discussion

In this study, we investigated the effects of SST on SI-induced behavioral and pharmacological abnormalities in mice, to explore therapeutic agents for patients with developmental disorders, including ADHD, using traditional Kampo medicines. The results demonstrated that SST administration significantly ameliorated deficits in sociability, attention deficit-like behavior, and fear memory. Moreover, the decrease in the expression levels of Egr-1 by SI stress was restored by SST administration. These findings suggest that SST may be useful as a therapeutic agent in the treatment of ADHD.

In this study, we found that, when given daily from week 2 after commencement of SI, SST at doses of 800 and 2400 mg/kg/day ameliorated SI-induced behavioral abnormality, which is likely to be relevant to symptoms of developmental disorders such as ADHD. The doses of SST used in the present study were approximately 3 and 10 times stronger than the daily dose for clinical treatment of patients, but these doses were close to the dosage of other herbal medicines and chemical agents which have been employed in preclinical studies reported by our and other research groups.[29,30] In our experiments, there were no changes in the body weight and motor ability of mice by SST (data not shown), suggesting that there is no side effect of SST at least in the range of the dose used in the present study. Considering these clinical features and dose ranges of SST, our findings provide further experimental evidence supporting the traditional application of SST for improvement of psychoneurotic symptoms in patients.

The mechanisms by which SST treatment attenuated SI-induced deficits in sociability and attention deficit-like behavior are unclear; however, several neuronal mechanisms are likely to be

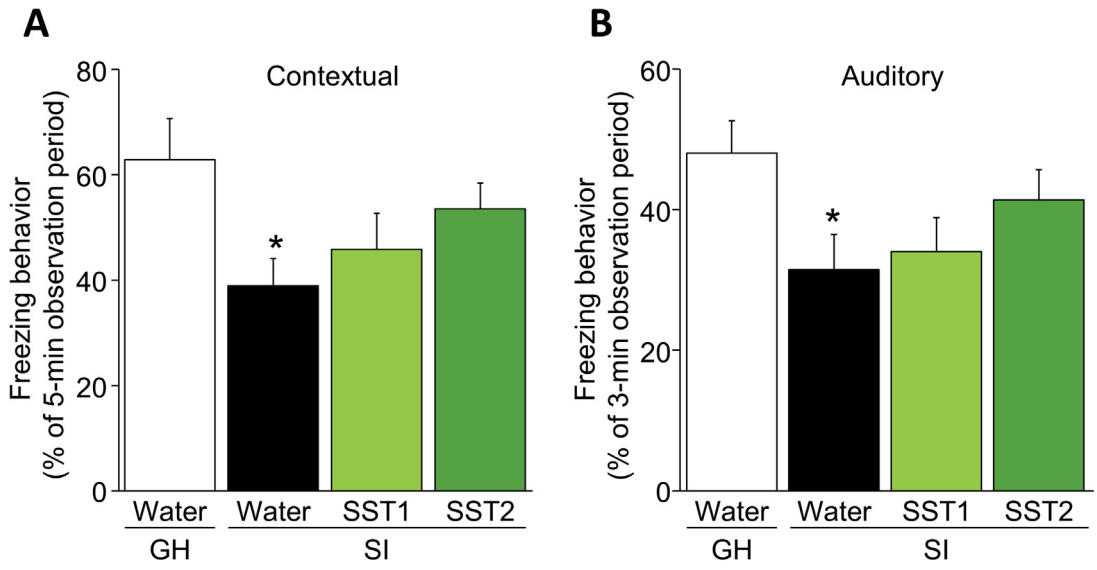

Fig. 4. The effect of SST on SI-induced long-term fear memory deficits in mice. Fear conditioning was conducted at the age of 11 weeks. Contextual (A) and auditory (B) memories were assessed at 1 and 5 days after fear conditioning. SST was given orally at doses of 800 (SST1) and 2400 (SST2) mg/kg for 5 weeks before the test. Each data column represents mean ± S.E.M. of 11 mice. *$p < 0.05$ vs. the GH group.

involved in the action of SST. In previous studies, we demonstrated that methylphenidate-induced enhancement of dopaminergic and cholinergic mechanisms via a muscarinic receptor cascade ameliorated SI-induced impairments of sociability and attention-deficit like behavior, respectively.[14,15] Taken together with the present findings, the ameliorative effects of SST on developmental disorder-like behavioral abnormalities in SI mice are likely to be mediated, at least in part, by attenuation of dopaminergic and cholinergic dysfunction caused by SI.

This study cannot exclude the possible involvement of GABAergic systems in the effects of SST due to several reasons. First, lines of evidence suggest that impairment of GABAergic function plays an important role in the pathophysiology of patients with developmental disorders such as ADHD and ASD via inducing an imbalance between glutamatergic and GABAergic signaling[31,32] or inducing dysfunction of dopaminergic systems in the brain.[33] Interestingly, dysfunction of GABAergic systems has been observed in several animal models of ADHD.[34,35] In fact, previous studies showed that SI stress results in a decrease in brain allopregnanolone (3α, 5α-tetrahydroprogesterone), a positive allosteric modulator of GABA on GABAA receptors, and thereby induces dysfunction of GABAergic systems. The changes in GABAergic function are reportedly involved in elevated aggressiveness,[36] increased susceptibility to pentetrazol,[37] and reduced response to pentobarbital in SI mice.[38,39] Second, it has been reported that some chemical constituents such of Zizyphi Semen, an important crude drug included in the SST formula, has an anxiolytic-like effect via modulation of GABAergic neurotransmission in the brain.[27] Thus, a speculative explanation for the effects of SST is that it may enhance the function of GABAergic systems in the brain, and thereby improve SI-induced behavior abnormalities. Experiments to test this theory are currently under progress in this laboratory.

The present study revealed that although the daily administration of SST tended to ameliorate impairment of contextual and auditory fear memory induced by SI stress, it significantly reversed the down-regulated expression of Egr-1 in the hippocampus and cortex of SI animals. Egr-1, a transcription factor that regulates the transcription of late-response genes, is known to be implicated in long-term synaptic plasticity, a molecular biological basis of learning and memory.[40] In fact, evidence indicates that Egr-1 plays an important role in contextual fear memory reconsolidation in the hippocampus[41] as well as in auditory fear memory consolidation and reconsolidation in the lateral amygdala.[42] We previously reported that SI stress induced deficits in conditioned fear memory and the down-regulation of Egr-1 expression and that tacrine attenuated these behavioral and neurochemical alterations via endogenous acetylcholine-mediated stimulation of muscarinic receptors.[15,16] Considering a close linkage between Egr-1 expression and fear memory performance probably through cholinergic systems, the present results suggest that SST may have an ameliorative effect on the SI-induced deficit in conditioned fear memories, probably at a more appropriate dose range.

There are two hypotheses about the mechanism by which the administration of SST improves SI-induced Egr-1 down-regulation; one is that SST restores the expression level of Egr-1 by activating cholinergic neuronal function, and the other is that SST affects epigenetic regulation of Egr-1. The former is based on our previous study that the SI-induced Egr-1 diminution is improved by tacrine, but not methylphenidate, and the restoring effect of tacrine on the down-regulation of Egr-1 expression is also reversed by scopolamine.[15] The latter is based on several observations that SI stress causes epigenetic changes of neurodevelopmental disorder-related protein expression. Moreover, Egr-1 also increases by epigenetic regulation in rat hippocampal neurons.[43,44] Moreover, Egr-1 also increases by epigenetic regulation in rat hippocampal neuron.[45] However, it is still unknown how SST regulates the Egr-1 expression in our present study. Therefore, it should be focused on elucidating the mechanism of Egr-1 expression by the administration of SST in our future study.

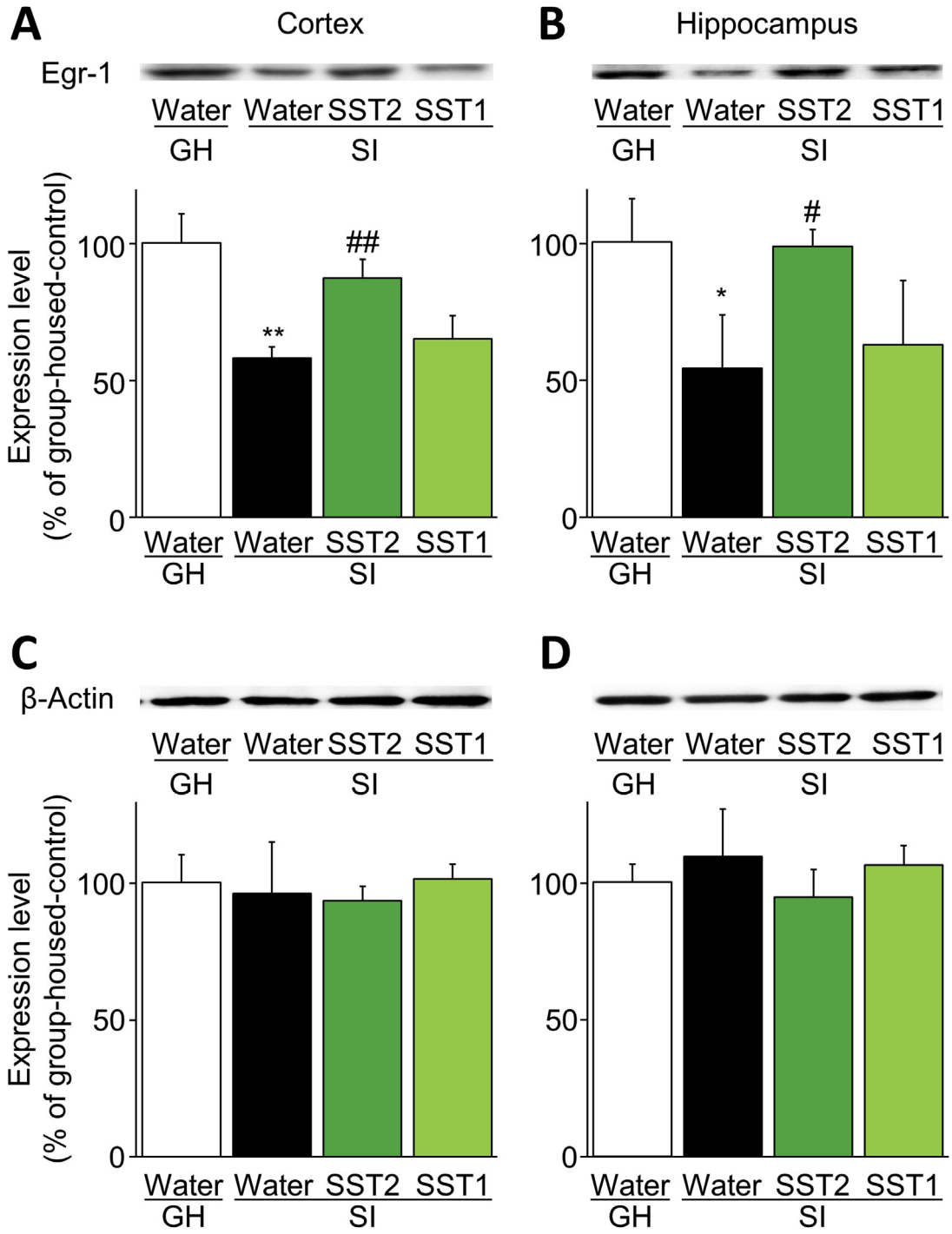

Fig. 5. Effects of SST on SI-induced down-regulated Egr-1 protein expression in the cortex and hippocampus of mice. Experiments were conducted after completing the auditory fear-conditioning test. Each animal was decapitated, and the frontal cortices and hippocampi were dissected for neurochemical studies. Typical photos indicate the expression levels of Egr-1 (A and B) and β-actin (a loading control; C and D) in the cortex (A and C) and hippocampus (B and D) obtained from group-housed (GH) and socially isolated mice (SI) treated with vehicle or SST [800 (SST1) and 2400 (SST2) mg/kg]. The densities of these bands were quantified and each data column represents mean ± S.E.M. obtained from four brain samples. *$p < 0.05$, **$p < 0.01$ vs. the GH group. #$p < 0.05$, ##$p < 0.01$ vs. the water-treated SI group.

5. Conclusion

This study indicates that daily administration of SST ameliorates SI-induced impairments of sociability, attention-like behavior, and expression of hippocampal Egr-1 relevant to fear memory. These results suggest that SST may be beneficial for the treatment of some symptoms in patients with developmental disorders such as ADHD.

Conflicts of interest

The authors and contributors of this work declare no conflicts of interest.

Acknowledgments

This study was in part supported by a Grant in-Aid for Challenging Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to KM (#24659347), a 2014 Presidential Grant for Strategic Research Promotion from University of Toyama (KM), a 2014 Grant-in-Aid from Kobayashi International Scholarship Foundation (KM), a Grant-in-Aid for the 2014 Cooperative Research Project I from the Institute of Natural Medicine, University of Toyama (KM) and a Grant-in-Aid for the 2016 Cooperative Research Project I from the Institute of Natural Medicine, University of Toyama (RK and HF).

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