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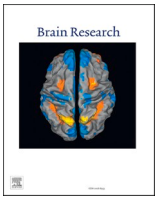

Kamishoyosan (a Japanese traditional herbal formula), which effectively reduces the aggressive biting behavior of male and female mice, and potential regulation through increase of *Tph1, Tph2,* and *Esr2* mRNA levels

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ABSTRACT

Kamishoyosan (KSS), a Japanese traditional herbal formula, is used to treat symptoms related to the autonomic nervous system in men and women; it is especially known for improving the symptoms of irritability (e.g., bad temper and persistent anger). Although clinical and ethological studies of KSS have been conducted, its efficacy in reducing irritability remains to be validated. In the present study, male and female ddY-strain mice were isolation-reared for 8 weeks (from the third postnatal week) to induce pathologically aggressive biting behavior (ABB), which was used as an indicator of irritability. The ABB of mice toward metal rods was measured using the Aggressive Response Meter. An intraperitoneal administration of KSS (100 mg/kg) effectively reduced ABB in male and female mice at 2 h after the administration; however, this effect was canceled by prior administration of WAY-100635 [a 5-hydroxytryptoamine (5-HT)-1A receptor antagonist; 0.5 mg/kg] and bicuculline (a type-A gamma-aminobutyric acid receptor antagonist; 1.0 mg/kg). Additionally, tamoxifen, ICI-182780, and G-15 (all estrogen receptor antagonists) inhibited the action of KSS in a dose-dependent manner. Furthermore, gene expression of *tryptophan hydroxylase* (*Tph*) *1* and *Tph2* were increased and 5-HT immunofluorescence was slightly increased in the dorsal raphe nucleus (DRN) of isolation-reared mice administered with KSS. Collectively, these results indicate that KSS effectively reduces ABB in isolation-reared male and female mice through stimulation of 5-HT production in the DRN. Our findings also suggest that gene expression of *estrogen receptor* (*Esr*) *2* increased in the DRN might be associated with the reduction of ABB.

1. Introduction

Kamishoyosan (KSS; Kami-shoyo-san or Jia-Wey Shiau-Yau San in Chinese) is a traditional herbal formula in Japan that is used to treat symptoms related to autonomic nervous system symptoms, such as palpitations, sweating, and hot flashes, as well as psychiatric-related symptoms, such as irritability (e.g., bad temper or persistent anger), insomnia, and general malaise. KSS is considered an effective supplement to improve these symptoms especially for men with some frailty and women (regardless of their constitution). Several clinical pilot studies have reported that KSS is an effective treatment for menstrual-related mood disorders, including premenstrual syndrome, premenstrual dysphoric disorder, and perimenopausal depression with climacteric symptoms (Chen et al., 2003; Hidaka et al., 2013; Yamada and

Abbreviations: ABB, aggressive biting behavior; ANOVA, analysis of variance; ARM, Aggressive Response Meter; DRN, dorsal raphe nucleus; ER, estrogen receptor; E2, estradiol; GABA, gamma-aminobutyric acid; i.p., intraperitoneal; KSS, Kamishoyosan; 5-HT, 5-hydroxytryptamine.

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Kanba, 2007). Case studies have reported that KSS is effective for treating female patient of panic disorder (Mantani et al., 2002; Sato-Nishimori et al., 2008). Recently, it was also reported that KSS improves women's excitability and irritability by a randomized controlled study (Takamatsu et al., 2021). In addition to above studies, it was also reported that KSS is effective for psychiatric symptoms of men. A case study was reported that KSS improved the symptoms of male patients with autonomic nervous system dysfunctions complaining of easy fatigability, palpitation, and so on (Matsuda, 1975). Another clinical study of Free and Easy Wanderer Plus (FEWP), a Chinese prescription similar to KSS, suggested that FEWP monotherapy effectively improved depressive symptoms in bipolar patients of both sexes (Zhang et al., 2007). These previous studies suggest that KSS exerts psychiatric action on both man and women, although the exact mechanistic action of KSS and any difference between sexes remains unclear. Animal (typically rodent) models have been used to investigate its anxiolytic action or promotion of prosocial behaviors; such studies help to understand the psychiatric action of KSS (Guo et al., 2019a, b; Mizowaki et al., 2001). Based on these findings, we hypothesized that KSS would also be an effective treatment for irritability, which can trigger impulsive and/or pathological aggression. To date, however, the efficacy of KSS on pathologically aggressive behavior has yet to be studied in detail. Thus, in the present study, we used an experimental mouse model to examine the efficacy of KSS against irritability or pathologically aggressive behavior.

A variety of psychiatric disorders include symptoms involving persistent anger and/or aggressive behavior (Matson and Jang, 2014; Patel and Barzman, 2013; Topiwala and Fazel, 2011; Tsiouris et al., 2011). Both genetic background and environmental factors, which can include mistreatment, harsh parenting, and impaired interpersonal relationships during development, may be associated with a tendency toward pathologically aggressive behavior (Waltes et al., 2016). Animals maintained under stress-rearing conditions can be used as experimental models to evaluate the causal relationships between environmental factors and aggressive behaviors. In particular, the maternal separation (MS) model and postweaning social isolation (PWSI) model are commonly applied to induce pathologically aggressive behavior in rodents (Haller et al., 2014). In representative MS experiments, pups are typically separated from both the dam and their littermates for 3–4 h per day to model adverse childhood experiences such as emotional neglect and social deprivation (Furukawa et al., 2017; Veenema et al., 2006). Numerous effects of MS have been described; they include not only excessive aggression but also anxiety-related behavior and impaired cognitive function (Cao et al., 2014; Wigger and Neumann, 1999). In representative PWSI experiments, male rats are usually reared in isolation after weaning (postnatal days 21–80) (Tóth et al., 2008). PWSI is considered a useful model for studying the developmental aspects of human reactive aggression, which often results from early social neglect.

Within the last decade, development of the Aggressive Response Meter (ARM) has improved measurements of aggressive behavior in male and female mice (Kuchiiwa and Kuchiiwa, 2014, 2016). In previous studies, the ARM was used to measure increasing aggressive biting behavior (ABB) of ddY-strain mice that were isolation-reared from 3 to 10 weeks after birth; ABB toward two metal rods was measured by a load sensor in the ARM (Kuchiiwa and Kuchiiwa, 2014, 2016). In these studies, healthy (group-housed) mice showed little or no aggressive behavior, whereas isolation-reared mice showed increasing ABB, which was considered a measurement of pathological aggression. In addition to measuring aggressive behavior in mice, the ARM device was intended to evaluate the effect of psychotropic drugs such as buspirone, which is a 5-hydroxytryptamine (5-HT)-1A receptor agonist that can function as an antidepressant.

In the present study, we examined the intensity and frequency of ABB in male and female mice, and we evaluated the effect of KSS on the irritability of these mice using the ARM. We first focused that the effect of KSS was due to its action on 5-HT_{1A} receptors, since agonistic action of buspirone on these receptors effectively reduced ABB in a previous study (Kuchiiwa and Kuchiiwa, 2014). In addition, it recently has been reported that KSS exerts antidepressive effects through 5-HT_{1A} receptor (Shimizu et al., 2019). To test this, we examined whether a 5-HT_{1A} receptor antagonist (WAY-100635) interfered with the effect of KSS. In addition, we hypothesized that estrogen receptors (ERs) were associated with the psychiatric action of KSS, since KSS is reported to act on ERβ (Kumagai et al., 2005; Watanabe et al., 2006). To test this, we applied ER antagonists (i.e., tamoxifen, ICI-182,780, and G-15) to determine whether this action on ERs was related to reduced ABB, and we investigated the amelioration of ABB by 17α- and 17β-estradiol (E2). Furthermore, we evaluated mRNA expression of the *tryptophan hydroxylase* gene (*Tph*; the tryptophan hydroxylase enzyme being rate-limiting within 5-HT synthesis) in brain tissue fragments of the dorsal raphe nucleus (DRN) and assessed 5-HT immunofluorescence in the DRN by quantitative real-time polymerase chain reaction (qRT-PCR) and immunofluorescence staining, respectively. Finally, we examined *estrogen receptor 2 (Esr2)* mRNA and ERβ protein expression.

2. Results

2.1. KSS-treated mice show reduced aggressive biting behavior

In the present study, male and female mice were reared in socially isolated conditions (i.e., lacking physical contact with other mice) from 3 weeks after their birth. Socially isolated mice were tested within the period during which they had received 6–8 weeks of isolation rearing (i.e., postnatal week 9–11). At postnatal week 10–11, the subject mouse is placed in the cylinder on the top of ARM and measured ABB. As in a previous study (Kuchiiwa and Kuchiiwa, 2014), metal rods with a dome-shape head are automatically controlled to make an up-and-down motion 30 times and the load sensor connected to the rods measures ABB intensity (average intensity of biting) and ABB frequency (total number of bitings within 30 presentations of the rods; see Supplementary Fig. 1). ABB intensity is expressed in numerical values as an integral of biting force within one-second presentation of the rods (milliNewton × second: mNs). In a previous study, isolation-reared mice showing a certain range of ABB intensity and intraperitoneal (i.p.) administration were used to examine the effect of buspirone on the aggressive behavior of isolation-reared mice (Kuchiiwa and Kuchiiwa, 2014). In the present study, we prescreened isolation-reared mice for their ABB intensity within 7–15 mNs, and investigated whether i.p. administration of KSS attenuated the ABB of isolation-reared mice (Fig. 1B–E). The dose of KSS was set at 100 mg/kg, which is not higher than the dose for human in a day. One-way analysis of variance (ANOVA) showed that the intensity and frequency of ABB in mice administered with saline via i.p. injection were similar to those of mice tested before saline administration ($F_{(2,27)} = 0.76, p = 0.47$ in ABB intensity of saline-treated males, $F_{(2,27)} = 0.48$, $p = 0.63$ in ABB frequency of saline-treated males, $F_{(2,23)} = 0.12$, $p = 0.89$ in ABB intensity of saline-treated females, and $F_{(2,23)} = 0.45$, $p = 0.64$ in ABB frequency of saline-treated females). In contrast, one-way ANOVA showed statistically significant change in the intensity of ABB in both male and female mice intraperitoneally administered KSS ($F_{(2,25)} = 11.34, p = 0.0003$ in ABB intensity of KSS-treated males, $F_{(2,25)} = 5.67, p = 0.009$ in ABB frequency of KSS-treated males, $F_{(2,21)} = 14.18$, $p = 0.0001$ in ABB intensity of saline-treated females, and $F_{(2,21)} = 10.74$, $p = 0.0006$ in ABB frequency of KSS-treated females). *Post hoc* analysis revealed that ABB intensity significantly decreased at 1 and 2 h after injection when compared with the ABB of mice before administration ($p = 0.002$ at 1 h and $p = 0.001$ at 2 h in male mice; $p = 0.0002$ at 1 h and $p = 0.002$ in female mice). Similarly, the frequency of ABB in mice significantly decreased at 1 and 2 h after i.p. administration of KSS ($p = 0.02$ at 1 h and $p = 0.02$ at 2 h in male mice; $p = 0.0008$ at 1 h and $p = 0.07$ in female mice). These results indicated that i.p. administration of KSS effectively reduces the ABB of both male and female isolation-

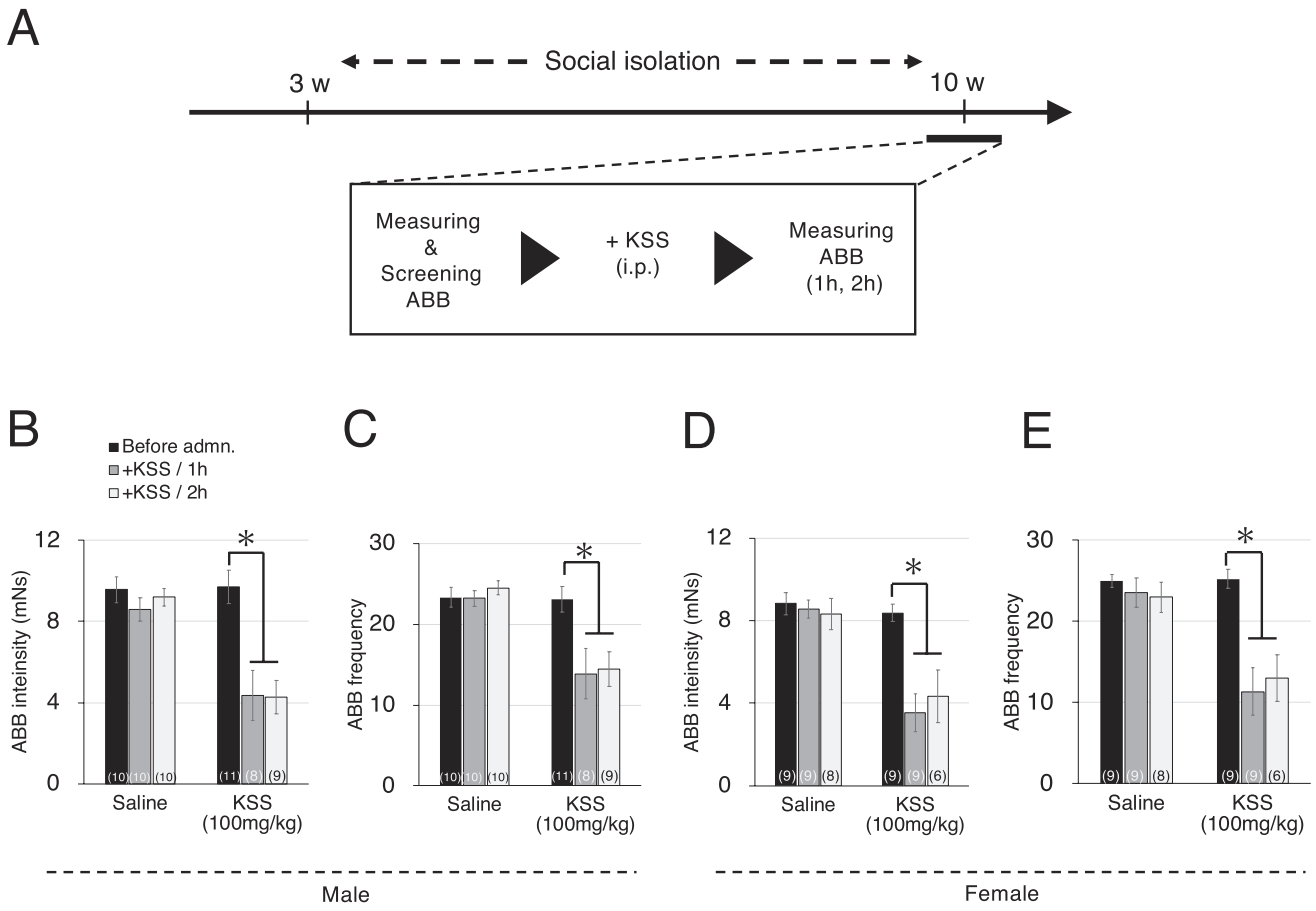

Fig. 1. Reduction of biting behavior in isolation-reared mice administered with Kamishoyosan (KSS). (A) Scheme of the experimental procedure. After 6–8 weeks of social isolation, the biting intensity of each mouse was measured to screen for mice with a biting intensity of 7–15 mNs. These mice were intraperitoneally administered with KSS extract or saline. At 1 and 2 h after administration, ABB intensity and ABB frequency (number of biting behaviors in measurement session) were measured. (B–E) The effect of KSS on the biting behavior of male (B, C) and female (D, E) mice. The intensity and frequency of ABB in these measurement sessions were respectively averaged within each experimental group; thus, data represent means ± standard errors. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (vs. preadministration; one-way ANOVA followed by Tukey–Kramer test).

reared mice. In isolation-reared female mice, we also confirmed that the effect of i.p. KSS administration on ABB was not dependent on their estrous cycle (see Supplementary Fig. 2). Thus, we did not examine estrous cycle of female mice in the following experiments.

We then examined whether a single oral administration of KSS (100, 200, and 400 mg/kg) through a plastic sonde attenuated the ABB of isolation-reared male and female mice (Fig. 2), since KSS is orally administered for human patients. One-way ANOVA showed no statistical significance within saline treatment group ($F_{(2,9)} = 0.51$, $p = 0.62$ in saline-treated males and $F_{(2,12)} = 0.86$, $p = 0.45$ in saline-treated females). In contrast, a statistical significance was found within male mice orally treated with 400 mg/kg of KSS ($F_{(2,6)} = 12.12$, $p = 0.0078$). *Post hoc* analysis showed a significant decrease in ABB intensity at 1 and 2 h after oral administration ($p = 0.014$ at 1 h and $p = 0.011$ at 2 h). In addition, there was statistically significant change found in female mice orally treated with 200 mg/kg KSS and those with 400 mg/kg KSS ($F_{(2,9)} = 10.19$, $p = 0.0049$ in 200 mg/kg, and $F_{(2,12)} = 4.80$, $p = 0.029$ in 400 mg/kg). *Post hoc* analysis revealed a significant decrease in biting intensity at 1 and 2 h after oral administration ($p = 0.032$ at 1 h and $p = 0.0045$ at 2 h in 200 mg/kg KSS treated female; $p = 0.11$ at 1 h and $p = 0.028$ at 2 h in 400 mg/kg KSS treated female). However, no significant effect was observed in ABB frequency at any of the three doses in both male and female mice. From these observations, we considered i.p. administration is more sensitive application to measure the effect of KSS and applied it in the following experiments.

2.2. Action of KSS was less effective in group-housed mice

We administrated KSS to group-housed mice to compare its psychiatric action in isolation-reared and group-housed treatments (Fig. 3). Group-housed mice showed smaller ABB intensity and frequency, compared with those used as isolation-reared mice. The intensity and frequency of ABB in group-housed mice did not show a statistically significant difference between before and after KSS (100 mg/kg) i.p. administration ($F_{(2,15)} = 0.66$, $p = 0.53$ in ABB intensity of KSS-treated males; $F_{(2,15)} = 1.27$, $p = 0.31$ in ABB frequency of KSS-treated males; $F_{(2,6)} = 3.26$, $p = 0.11$ in ABB intensity of KSS-treated females; $F_{(2,6)} = 0.96$, $p = 0.43$ in ABB frequency of KSS-treated females), suggesting that KSS was less effective in subjects without pathological aggression.

2.3. KSS action was blocked by a 5-HT_{1A} receptor antagonist in female and in males by combinatory use of a 5-HT_{1A} receptor antagonist and a $GABA_A$ receptor antagonist

To test our hypothesis that 5-HT_{1A} receptors play some role in KSS-mediated amelioration of ABB, mice were pretreated with WAY-100635, a 5-HT_{1A} receptor antagonist (Fig. 4A). One-way ANOVA showed a statistical significance in the effect on ABB intensity ($F_{(2,15)} = 6.78$, $p = 0.008$) and on ABB frequency ($F_{(2,15)} = 11.30$, $p = 0.001$) after WAY-100635 and KSS administration on male mice (Fig. 4B and C). *Post hoc* analysis revealed that ABB intensity of male mice after KSS treatment was significantly decreased ($p = 0.02$ vs. before administration,

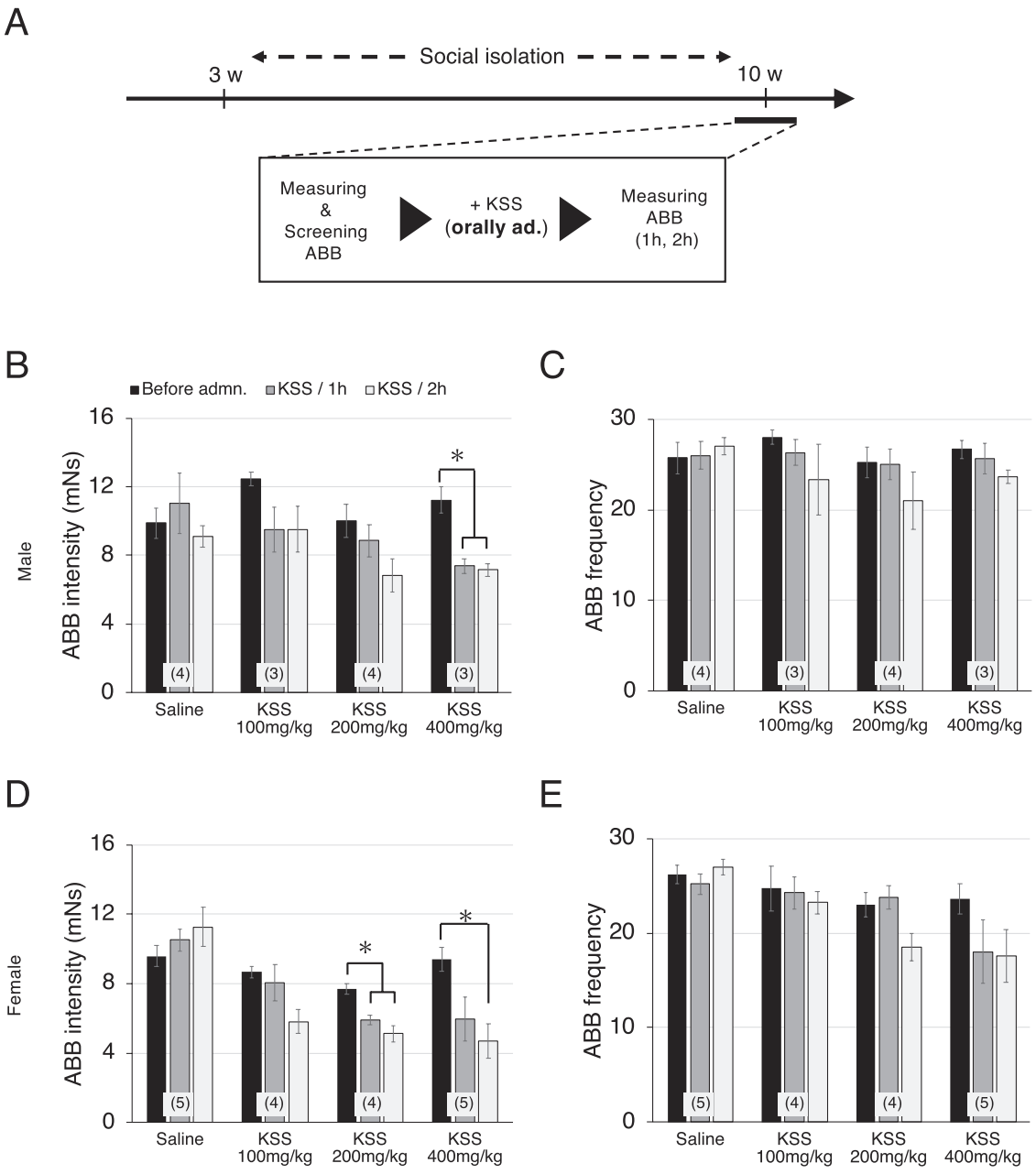

Fig. 2. Reduced aggressive biting behavior in mice orally administered with Kamishoyosan (KSS). (A) Scheme of the experimental procedure. ABB intensity of male (B) and female (D) mice. ABB frequency (Number of biting behavior in measurement session) of male (C) and female (E) mice. Error bars represent standard errors. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (one-way ANOVA followed by Tukey's HSD test).

and $p = 0.01$ vs. after WAY-100635 treatment), and that ABB frequency of male mice after KSS treatment was significantly decreased ($p = 0.003$ vs. before administration, and $p = 0.003$ vs. after WAY-100635 treatment). Thus, it was suggested that the action of KSS on ABB was not canceled by inhibition of 5-HT_{1A} receptors in male mice. Since it was previously shown that the action of KSS on social behavior involves the type-A gamma-aminobutyric acid ($GABA_A$) receptor (Guo et al., 2019a, 2019b), we also tested pretreatment of male mice with bicuculline, a $GABA_A$ receptor antagonist (Fig. 4B and C). The intensity and frequency of ABB in male mice treated with KSS following bicuculline significantly decreased after administration ($F_{(2,15)} = 6.81$, $p = 0.0079$ in ABB intensity of male mice, and $F_{(2,15)} = 19.93$, $p = 0.00006$ in ABB frequency of male mice), suggesting that the action of KSS on ABB was not canceled by inhibition of the $GABA_A$ receptor in male mice. In addition, we tested a treatment of KSS following WAY-100635 and bicuculline in male mice (Fig. 4B and C). We found that the intensity of ABB in these mice did not significantly decrease after administration ($F_{(2,21)} = 2.47$, $p = 0.11$) and that the frequency of ABB in male mice was not significantly affected by KSS following WAY-100635 and bicuculline pretreatment ($F_{(2,21)} = 0.59$, $p = 0.56$). These results suggest that the action of KSS on ABB was partly canceled by concomitant inhibition of the 5-HT_{1A} and $GABA_A$ receptors in male mice.

In contrast, statistically significant change was not shown in ABB intensity or frequency of female mice treated with KSS following WAY-100635 by one-way ANOVA ($F_{(2,20)} = 1.94$, $p = 0.17$ in ABB intensity of male mice and $F_{(2,20)} = 1.32$, $p = 0.29$ in ABB frequency of female mice; Fig. 4D and E), suggesting that the action of KSS on ABB was canceled by inhibition of 5-HT_{1A} receptors in female mice. We also examined the effect of bicuculline preadministration and following KSS administration, and statistically significant change was shown by one-way ANOVA

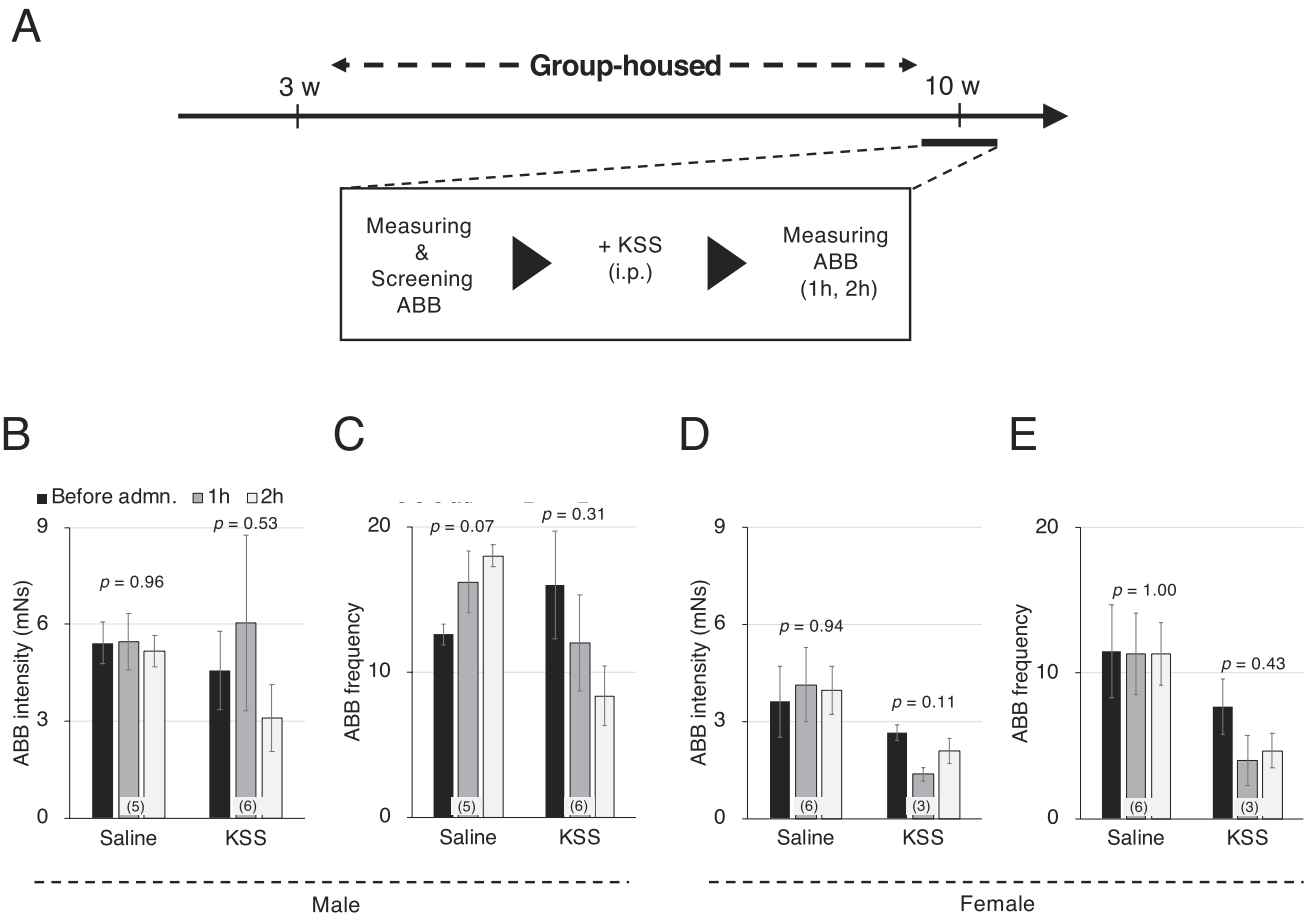

Fig. 3. Kamishoyosan (KSS) had no significant effect on aggressive biting behavior in group-housed mice. (A) Scheme of the experimental procedure. ABB intensity of group-housed male (B) and female (D) mice. ABB frequency of group-housed male (C) and female (E) mice. Error bars represent standard errors. Numbers in brackets represent numbers of mice used in each experimental group. Data were analyzed using one-way ANOVA and statistical significance at $p < 0.05$ was not detected.

($F_{(2,15)} = 26.37$, $p = 0.00001$ in ABB intensity, and $F_{(2,15)} = 19.98$, $p = 0.00006$ in ABB frequency), suggesting that the action of KSS on ABB was not canceled by inhibition of the $GABA_A$ receptor in female mice. We further examined whether the combinatory preadministration of WAY-100635 and bicuculline affects ABB of female mice, and no statistically significant change was shown in both ABB intensity and frequency by one-way ANOVA ($F_{(2,6)} = 0.36$, $p = 0.71$ in ABB intensity, and $F_{(2,6)} = 3.08$, $p = 0.12$ in ABB frequency).

2.4. KSS action is blocked by estrogen receptor antagonists

Since KSS is used to mitigate the symptoms of menopausal syndromes, such as hot flashes and dysphoria, in combination with hormone replacement therapy (Hidaka et al., 2013), we hypothesized whether KSS action on estrogen receptors (ER) has a certain association with reducing ABB. In order to investigate the hypothesis, we used tamoxifen and ICI-182,780 as antagonists for classical ERs (ERα and ERβ), and G-15 as an antagonist for GPR30 (a 7-transmembrane G protein-coupled ER) (Prossnitz et al., 2008; Shanle and Xu, 2010). Isolation-reared mice were preadministered with ER antagonists (tamoxifen, ICI-182,780, or G-15) by i.p. injection 30 min before administration of 100 mg/kg KSS by i.p. injection. Statistically significant changes within the ABB intensity of male and female mice pretreated with vehicle was shown by one-way ANOVA ($F_{(2,6)} = 10.65$, $p = 0.01$ in males and $F_{(2,6)} = 9.62$, $p = 0.01$ in females). *Post hoc* analysis revealed reduction of ABB intensity after administration of KSS by about 56% in males ($p = 0.02$ vs. before administration, and $p = 0.01$ vs. after vehicle treatment) and 63% in females ($p = 0.02$ vs. before administration, and $p = 0.02$ vs. after vehicle); however, the ABB intensity of male and female mice that were pretreated with tamoxifen (5.0 mg/kg), ICI-182,780 (5.0 mg/kg), or G-15 (0.35 mg/kg) before KSS administration did not show statistically significant change ($F_{(2,9)} = 0.07$, $p = 0.94$ in tamoxifen (5.0 mg/kg)-treated male, $F_{(2,6)} = 1.05$, $p = 0.41$ in tamoxifen (5.0 mg/kg)-treated female, $F_{(2,9)} = 0.97$, $p = 0.41$ in ICI-182,780 (5.0 mg/kg)-treated male, $F_{(2,6)} = 0.84$, $p = 0.48$ in ICI-182,780 (5 mg/kg)-treated female, $F_{(2,9)} = 2.60$, $p = 0.13$ in G-15 (0.35 mg/kg)-treated male, $F_{(2,9)} = 0.09$, $p = 0.92$ in G-15 (0.35 mg/kg)-treated female; Fig. 5B and D). One-way ANOVA analysis also showed statistical significant changes in the frequency of ABB in male and female mice pretreated with vehicle ($F_{(2,6)} = 7$, $p = 0.027$ in male and $F_{(2,6)} = 6.19$, $p = 0.035$ in female), and *post hoc* analysis revealed reduction of ABB intensity after administration of KSS in males ($p = 0.04$ vs. before administration, and $p = 0.04$ vs. after vehicle treatment) and in females ($p = 0.04$ vs. before administration, and $p = 0.04$ vs. after vehicle). On the other hand, statistically significant changes were not found within the frequency of ABB in male and female mice pretreated with tamoxifen, ICI-182,780, or G-15 ($F_{(2,9)} = 1.19$, $p = 0.34$ in tamoxifen (5.0 mg/kg)-treated male, $F_{(2,6)} = 0.016$, $p = 0.98$ in tamoxifen (5.0 mg/kg)-treated female, $F_{(2,9)} = 0.80$, $p = 0.48$ in ICI-182,780 (5.0 mg/kg)-treated male, $F_{(2,6)} = 0.50$, $p = 0.63$ in ICI-182,780 (5.0 mg/kg)-treated female, $F_{(2,9)} = 0.33$, $p = 0.72$ in G-15 (0.35 mg/kg)-treated male, $F_{(2,9)} = 0.13$, $p = 0.88$ in G-15 (0.35 mg/kg)-treated female; Fig. 5C and E). These results suggest that KSS acted in some manner on ER to reduce ABB in both male and female mice.

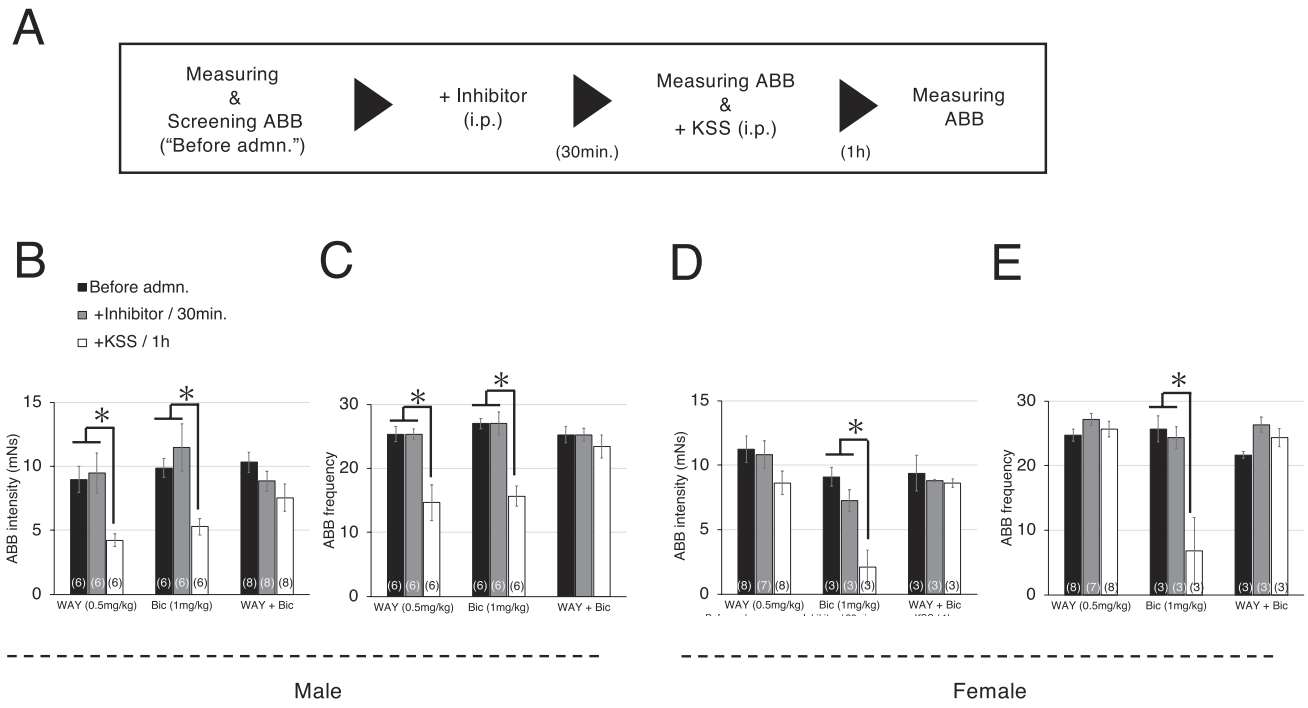

Fig. 4. Preadministration of the 5-HT_{1A} serotonin receptor inhibitor WAY-100635 abolished the Kamishoyosan (KSS)-induced reduction in biting behavior in isolation-reared female mice, while in males this blocking effect was only observed when the $GABA_A$ receptor antagonist bicuculline was also present. (A) Scheme of the experimental procedure. After 6–8 weeks of social isolation, the biting intensity of each mouse was measured to screen for mice with a biting intensity of 7–15 mNs. These mice were intraperitoneally administered with the 5-HT_{1A} serotonin receptor inhibitor WAY-100635 (WAY; 0.5 mg/kg) and/or the $GABA_A$ receptor inhibitor bicuculline (Bic; 1 mg/kg). Thirty minutes after administration of inhibitors, ABB intensity and ABB frequency were measured, and then mice were intraperitoneally administered with KSS (100 mg/kg). At 1 h after KSS administration, the intensity and frequency were measured. (B–E) The biting behaviors of male (B, C) and female (D, E) mice. The intensity and frequency of ABB in these measurement sessions were respectively averaged within each experimental group; thus, data represent means ± standard errors. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (one-way ANOVA followed by Tukey's HSD test).

2.5. ABB is reduced in mice administered with 17β-E2

Since it has been reported that 1 g of KSS exerts estrogen-like activity equivalent to 13.3 pg of 17β-E2 in a luciferase reporter assay (Kumagai et al., 2005), we examined whether the equivalent amount of 17α- or 17β-E2 would reduce ABB in isolation-reared mice at 1 h after the administration, as KSS exerted the ABB-reducing effect at 1 h after the administration. The ABB intensity of mice administered with 17β-E2 was reduced after treatment by about 46% in males ($p = 0.02$) and about 29% in females ($p = 0.006$), whereas the ABB intensity of mice of both sexes administered with 17α-E2 did not differ significantly before and after treatment ($p = 0.21$ in males and $p = 0.08$ in females; Fig. 6B and D). The frequency of ABB in male mice administered with 17β-E2 was also significantly reduced after treatment, but it did not differ before and after treatment in females ($p = 0.049$ in males and $p = 0.52$ in females; Fig. 6C and E). Collectively, these results support the hypothesis that ERs are partly involved in the ABB-reducing action of KSS.

2.6. Tph1 and Tph2 mRNA increases in DRN of KSS-treated mice

A large proportion of serotonin in the brain is produced by a major group of serotonergic neurons in the DRN of the midbrain (Dahlström and Fuxe, 1964; Hensler, 2006). To investigate gene expression of *Tph*, which encodes a rate-limiting enzyme during 5-HT synthesis, we performed qRT-PCR analysis on DRN fragments sampled from isolation-reared mice 2 h after administration of KSS (Fig. 7B and C). Since *Tph* has two isoforms, *Tph1* and *Tph2*, we examined respective mRNA levels of each isoform. Two-way ANOVA showed a significant effect of the treatment on *Tph1* mRNA level ($F_{(1,8)} = 40.80$, $p = 0.0002$) and of the sex ($F_{(1,8)} = 40.72$, $p = 0.0002$) with no interaction effect ($F_{(1,8)} = 0.0036$, $p = 0.95$). *Post hoc* analysis revealed increases of *Tph1* mRNA level in KSS-treated male mice ($p = 0.0022$) and KSS-treated female mice ($p = 0.0020$). In addition, a statistically significant difference was found between saline-treated male and saline-treated female mice ($p = 0.0020$). Two-way ANOVA analysis was also showed a significant effect of the treatment on *Tph2* mRNA level ($F_{(1,8)} = 89.75$, $p = 0.00001$) and of the sex ($F_{(1,8)} = 136.74$, $p = 2.6 \times 10^{-6}$) with significant interaction effect ($F_{(1,8)} = 23.06$, $p = 0.0014$). *Post hoc* analysis revealed increases of *Tph2* mRNA level in KSS-treated male mice ($p = 0.0002$) and KSS-treated female mice ($p = 0.011$). In addition, a statistically significant difference was found between saline-treated male and saline-treated female mice ($p = 0.0002$).

To examine 5-HT production in the DRN of mice brains following administration of KSS, we also performed immunofluorescence staining using an anti-5-HT antibody (Fig. 7D and E). A characteristic pattern was confirmed, which has been reported in a previous study (VanderHorst et al., 2005). Two-way ANOVA analysis showed a significant effect of the treatment on immunofluorescence intensity ($F_{(1,8)} = 6.52$, $p = 0.034$) and of the sex ($F_{(1,8)} = 28.92$, $p = 0.0007$) with no interaction effect ($F_{(1,8)} = 0.21$, $p = 0.66$). *Post hoc* analysis revealed a statistically significant difference was found between saline-treated male and saline-treated female mice ($p = 0.0085$). However, no significant increase was found in KSS-treated male mice ($p = 0.18$) or in KSS-treated female mice ($p = 0.066$).

It has been previously reported that prefrontal cortex (PFC) regulates intermale aggressive behavior and that PFC is one of the common brain regions innervated by the serotonergic projection from the middle part of the DRN, which we have examined here (Fernandez et al., 2016; Takahashi et al., 2014; Waselus et al., 2011). In order to further examine the efficacy of KSS administration, we thus examined *c-fos* gene

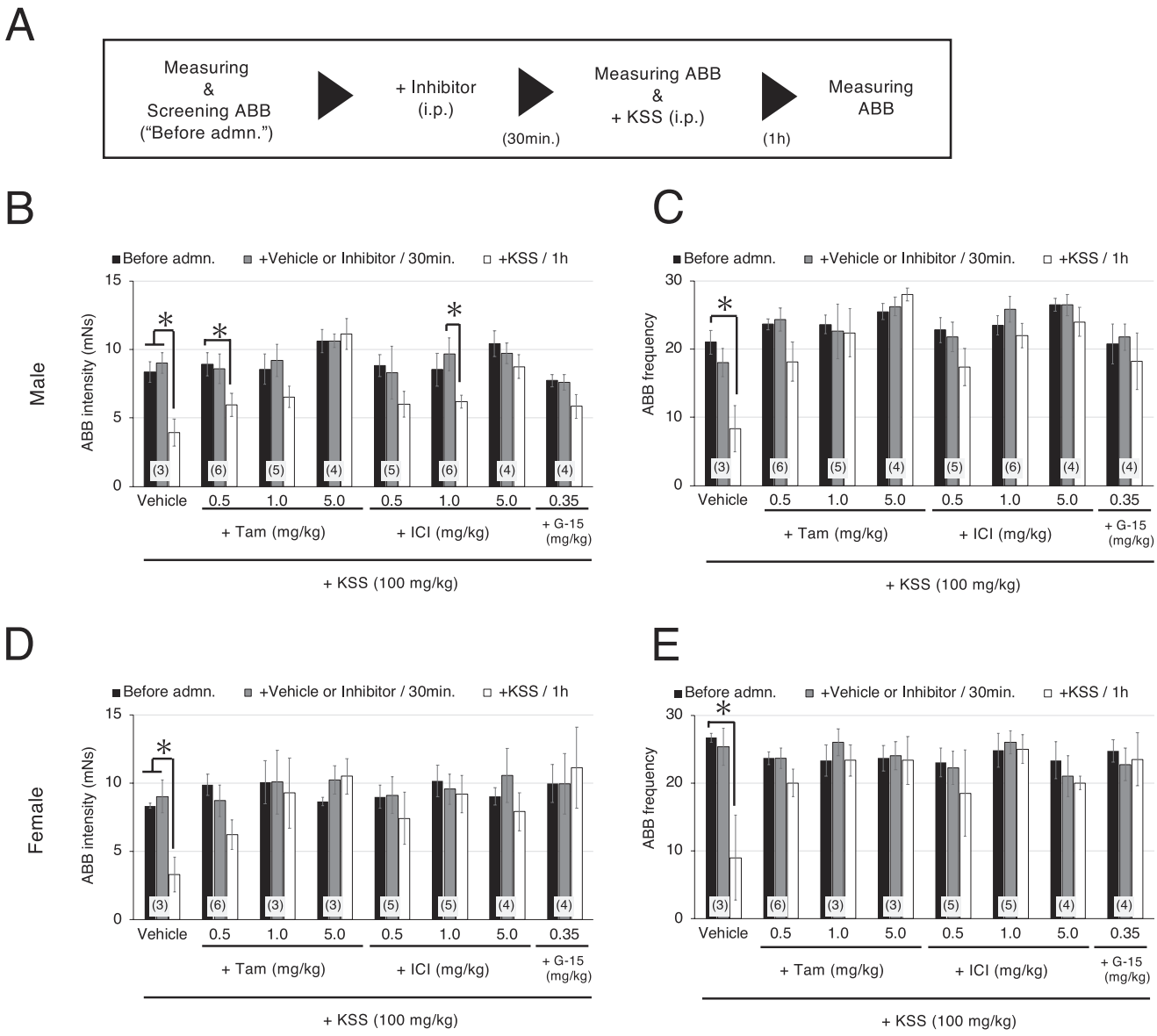

Fig. 5. Preadministration of estrogen receptor inhibitors abolished the Kamishoyosan (KSS)-mediated reduction in biting behaviors in isolation-reared mice. (A) Scheme of the experimental procedure. After 6–8 weeks of social isolation, the biting intensity of each mouse was measured to screen for mice with a biting intensity of 7–15 mNs. These mice were intraperitoneally administered with estrogen receptor inhibitor tamoxifen (Tam), ICI-182,780 (ICI), G-15, or vehicle. Thirty minutes after administration of inhibitors, ABB intensity and ABB frequency were measured, and then mice were intraperitoneally administered with KSS. At 1 h after KSS administration, ABB intensity and frequency were measured. (B–E) The biting behaviors of male (B, C) and female (D, E) mice. The intensity and frequency of biting behaviors in these measurement sessions were respectively averaged within each experimental group; thus, data represent means ± standard errors. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (one-way ANOVA followed by Tukey's HSD test).

expression in DRN and PFC after KSS administration (Fig. 7F and G). Two-way ANOVA of *c-fos* gene expression in DRN showed a significant effect of the treatment on *c-fos* mRNA level ($F_{(1,8)} = 506.8$, $p = 1.6 \times 10^{-8}$) and of the sex ($F_{(1,8)} = 1839$, $p = 9.6 \times 10^{-11}$) with significant interaction effect ($F_{(1,8)} = 161.4$, $p = 1.4 \times 10^{-6}$). *Post hoc* analysis revealed increases of *c-fos* mRNA level in KSS-treated male mice ($p = 0.0002$) and KSS-treated female mice ($p = 0.0003$). These results suggest that the level of neuroactivation in the DRN was decreased in the male and female mice that were administered with KSS. In addition, a statistically significant difference was found between saline-treated male and saline-treated female mice ($p = 0.0002$). Two-way ANOVA of *c-fos* gene expression in PFC showed no significant effect of the treatment on *c-fos* mRNA level ($F_{(1,8)} = 1.03$, $p = 0.34$) and of the sex ($F_{(1,8)} = 42.76$, $p = 0.0002$) with significant interaction effect ($F_{(1,8)} = 74.64$, $p = 0.00003$). *Post hoc* analysis revealed increase of *c-fos* mRNA level in KSS-treated male mice ($p = 0.0008$) and decrease of *c-fos* mRNA level in KSS-treated female mice ($p = 0.0003$). These results suggest that the level of neuroactivation in the PFC was not decreased in the male, while it was decreased in female mice. Statistically significant difference was not found between saline-treated male and saline-treated female mice ($p = 0.18$).

2.7. Estrogen receptors are associated with Tph2 mRNA induction in the DRN of male mice that were administrated with KSS

From above results, we surmised that ERs are associated with the action of *Tph1* and *Tph2* mRNA induction. We examined *Tph1* and *Tph2* mRNA expression in the DRN of the mice that were administrated with tamoxifen (5.0 mg/kg) or ICI-182,780 (5.0 mg/kg), followed by KSS treatment (Fig. 8). Statistically significant changes were not found

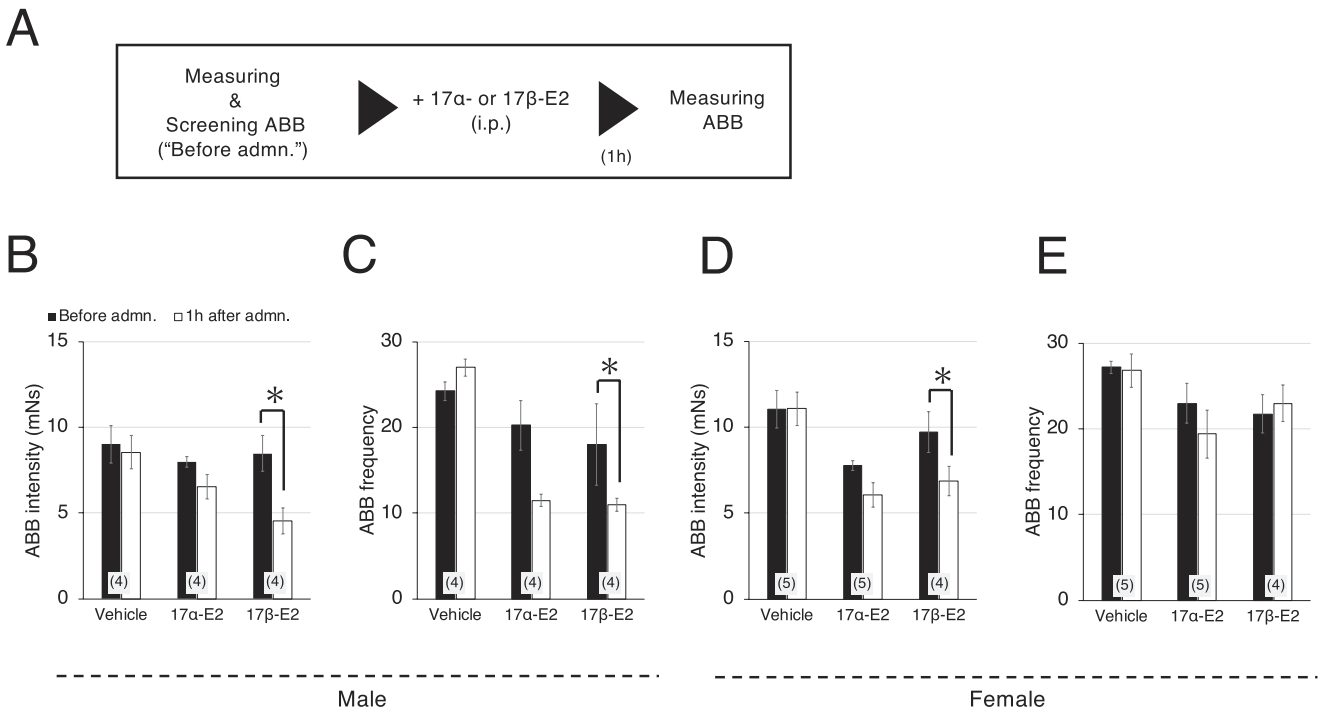

Fig. 6. The effects of 17α-E2 and 17β-E2 on the biting behavior of isolation-reared female and male mice. (A) Scheme of the experimental procedure. After 6–8 weeks of social isolation, the biting intensity of each mouse was measured to screen for mice with a biting intensity of 7–15 mNs. These mice were intraperitoneally administered with 17α- or 17β-E2 (1.33 pg/kg). One hour after administration of 17α- or 17β-E2, ABB intensity and ABB frequency were measured. (B–E) Biting behaviors in male (B, C) and female (D, E) mice. The intensity and frequency of biting behaviors in these measurement sessions were respectively averaged within each experimental group; thus, data represent means ± standard errors. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (Student's *t* test).

within *Tph1* mRNA expression in the DRN of male and female mice ($F_{(2,6)} = 0.34$, $p = 0.73$ in males; $F_{(2,6)} = 2.20$, $p = 0.19$ in females). In contrast, statistically significant changes were found within *Tph2* mRNA expression in the DRN of male and female mice ($F_{(2,6)} = 81.2$, $p = 0.00005$ in males; $F_{(2,6)} = 36.8$, $p = 0.0004$ in females). *Post hoc* analysis revealed decrease of *Tph2* mRNA expression in male mice administered with tamoxifen or ICI-182,780 ($p = 0.0002$ in comparison between tamoxifen and vehicle, $p = 0.0002$ in comparison between ICI and 182,780 and vehicle), suggesting that ERs are associated with *Tph2* mRNA induction in the DRN of male mice administered with KSS. It was also revealed that *Tph2* mRNA expression increases in female mice administered with tamoxifen or ICI-182,780 ($p = 0.0008$ in comparison between tamoxifen and vehicle, $p = 0.0009$ in comparison between ICI and 182,780 and vehicle).

2.8. *Esr2 (ERβ) mRNA levels increases in brain tissue fractions of the DRN and ERβ are involved with the ABB-reducing action of KSS*

From the above results, it was surmised that ERs are associated with the action of *Tph2* mRNA induction in the DRN of mice administered with KSS. Since it was previously reported that ERβ regulates *Tph2* mRNA expression in the DRN (Donner and Handa, 2009), we focused on *Esr2* (which encodes ERβ) mRNA expression, and performed qRT-PCR analysis on brain tissue fractions of the DRN sampled at 2 h after the administration of KSS or saline (Fig. 9B). Two-way ANOVA analysis was also showed a significant effect of the treatment on *Esr2* mRNA level ($F_{(1,8)} = 34.06$, $p = 0.0004$) but no significant effect of the sex ($F_{(1,8)} = 4.35$, $p = 0.07$) with no interaction effect ($F_{(1,8)} = 0.0023$, $p = 0.96$). *Post hoc* analysis revealed increases of *Esr2* mRNA level in KSS-treated male mice ($p = 0.0033$) and KSS-treated female mice ($p = 0.036$).

In addition, we used immunoblot analysis to investigate ERβ protein expression in isolation-reared mice 2 h after an i.p. administration of KSS or saline. Male mice administered with KSS showed a statistically significant increase ($p = 0.04$) in ERβ protein levels compared with male mice administered with saline (Fig. 9C). Female mice administered with KSS showed an increase in ERβ protein levels compared with female mice administered with saline, although a statistically significant increase was not detected ($p = 0.07$; Fig. 9D).

In order to validate whether ERβ are associated with the ABB-reducing action of KSS, isolation-reared mice were preadministered with an ERβ-selective antagonist (PHTPP) by i.p. injection 30 min before administration of 100 mg/kg KSS by i.p. injection (Fig. 9E–I). Statistically significant changes were not found within the intensity of ABB in male and female mice pretreated with PHTPP ($F_{(2,6)} = 1.71$, $p = 0.25$ in PHTPP (0.5 mg/kg)-treated male; $F_{(2,6)} = 0.02$, $p = 0.98$ in PHTPP (1.0 mg/kg)-treated male; $F_{(2,6)} = 2.37$, $p = 0.17$ in PHTPP (0.5 mg/kg)-treated female; $F_{(2,6)} = 0.13$, $p = 0.88$ in PHTPP (1.0 mg/kg)-treated female). No statistically significant change was found either within the frequency of ABB in male and female mice pretreated with PHTPP ($F_{(2,6)} = 2.40$, $p = 0.17$ in PHTPP (0.5 mg/kg)-treated male; $F_{(2,6)} = 2.13$, $p = 0.20$ in PHTPP (1.0 mg/kg)-treated male; $F_{(2,6)} = 0.96$, $p = 0.43$ in PHTPP (0.5 mg/kg)-treated female; $F_{(2,6)} = 0.29$, $p = 0.77$ in PHTPP (1.0 mg/kg)-treated female). These results suggest that KSS acted in some manner on ERβ to reduce ABB in both male and female mice.

3. Discussion

In the present study, we used an ARM device to evaluate the irritability of isolation-reared mice based on their propensity to aggressively bite metal rods. Our results indicate that administration of KSS effectively reduces the ABB of both male and female mice. Although KSS is conventionally used as an effective treatment for menopause-related psychological disorders and is also considered an effective supplement for men with autonomic nervous system dysfunctions (easy fatigability, palpitation and so on), the molecular basis for the action of KSS has yet to be fully elucidated (Hidaka et al., 2013; Matsuda, 1975). It is likely

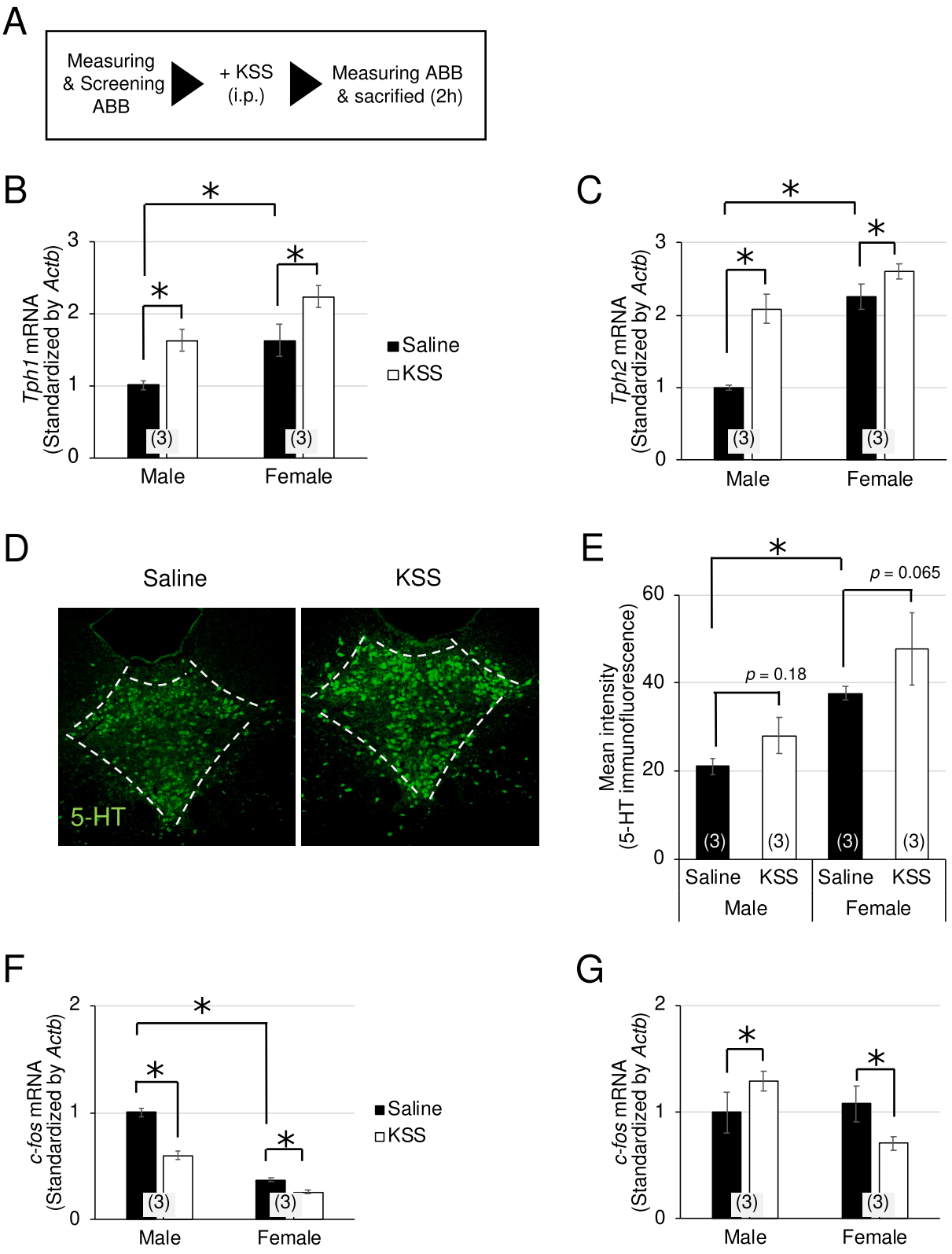

Fig. 7. *Tph1* and *Tph2* mRNA expression and 5-HT immunofluorescence in brain tissue fractions of the dorsal raphe nucleus (DRN). (A) Scheme of the experimental procedure. Isolation-reared mice were intraperitoneally administered with Kamishoyosan (KSS; 100 mg/kg) or saline, and brain tissue fractions of the DRN were resected at 2 h after the administration. (B, C) Expression levels of *Tph1* and *Tph2* according to qRT-PCR; data were normalized to *Actb* mRNA expression and shown as relative values compared with expression levels in male mice administered with saline. (D) Representative images of immunofluorescence staining using anti-5-HT antibody. (E) Fluorescence intensity ere measured in each mouse. (F, G) Expression levels of *c-fos* in DRN (F) and in prefrontal cortex (G) according to qRT-PCR; data were normalized to *Actb* mRNA expression and shown as relative values compared with expression levels in male mice administered with saline. Error bars represent standard deviations. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (two-way ANOVA followed by Tukey's HSD test).

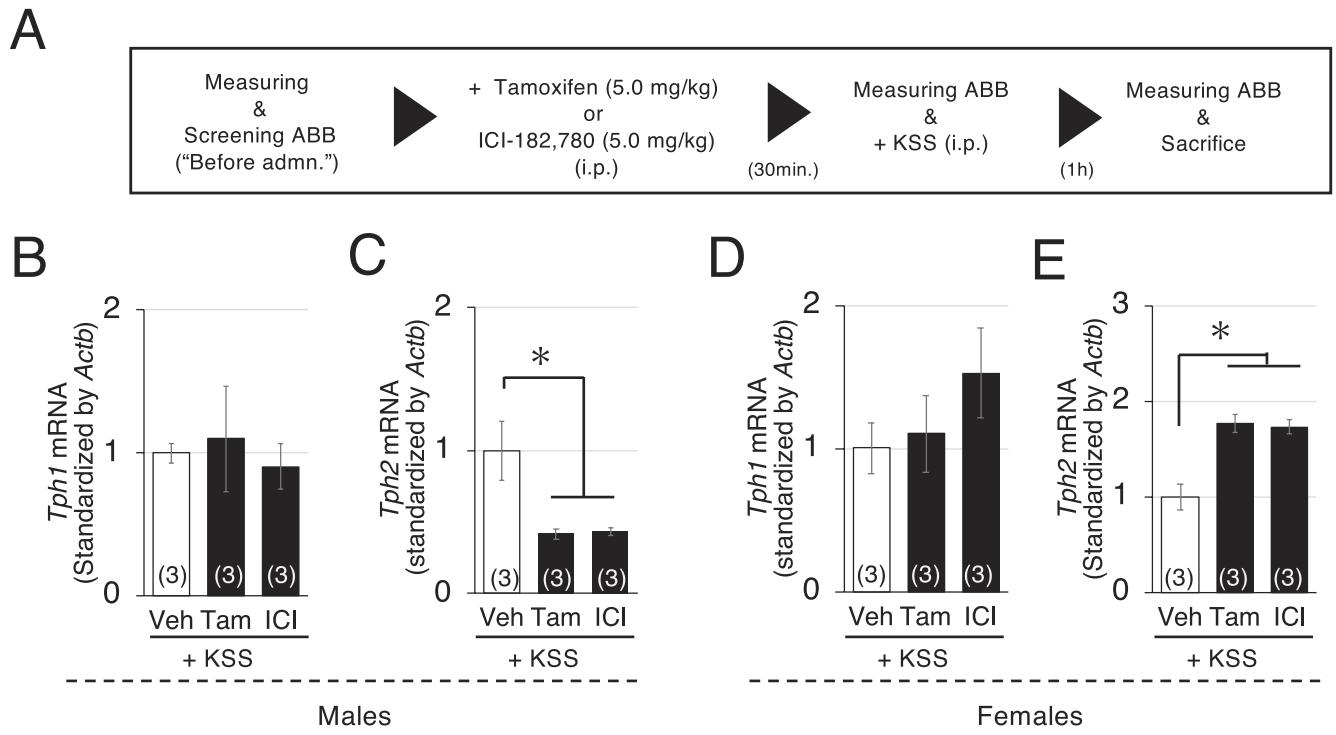

Fig. 8. *Tph1* and *Tph2* mRNA expression in the dorsal raphe nucleus (DRN) of the mice that were administrated with tamoxifen (5.0 mg/kg) or ICI-182,780 (5.0 mg/kg), and following KSS (100 mg/kg) treatment. Isolation-reared mice were intraperitoneally administered with tamoxifen (5.0 mg/kg), ICI-182,780 (5.0 mg/kg), or vehicle. Thirty minutes after the administration of these ER inhibitors, the mice were intraperitoneally administered with Kamishoyosan (KSS; 100 mg/kg), and brain tissue fractions of the DRN were resected at 1 h after the KSS administration. (B–E) Expression levels of *Tph1* (B, D) and *Tph2* (C, E) according to qRT-PCR; data were normalized to *Actb* mRNA expression and shown as relative values compared with expression levels in male (B, C) or female (D, E) mice administered with vehicle. Error bars represent standard deviations. Numbers in brackets represent numbers of mice used in each experimental group. * $p < 0.05$ (one-way ANOVA followed by Tukey's HSD test).

that multiple components are acting synergistically during KSS treatment, since traditional herbal formulas are complex and contain various crude ingredients. Our results indicated that KSS acts on 5-HT_{1A} receptors, leading to reduced aggressiveness in socially isolated male and female mice. In addition, KSS action on both 5-HT_{1A} receptors, and $GABA_A$ receptors seemed to be involved in reduced ABB in male mice. We also found that *Tph1* and *Tph2* induction after KSS treatment in the DRN. Our findings also suggest that increase of ERs in the DRN might be associated with reduction of ABB.

We showed that KSS reduced aggressive behavior in isolation-reared mice; previously, buspirone, a psychotic drug acting on 5-HT_{1A} receptors, was also shown to reduce aggressive behavior in mice reared in isolation (Kuchiiwa and Kuchiiwa, 2014). Our study included examination of the DRN, which is a major source of 5-HT in the brain and is functionally associated with emotional behaviors such as aggression (Azmitia et al., 2011; Dahlström and Fuxe, 1964; Faccidomo et al., 2008; Sijbesma et al., 1991; Vergnes et al., 1986). Specifically, we observed an increase in *Tph1* and *Tph2* gene expression in the DRN of mice administered with KSS. We also observed that *Tph1* and *Tph2* mRNA levels in females were higher compared with those in males. In a previous study, they performed restraint stress on pregnant rats and found that *Tph2* gene expression in the DRN of male offspring rats was decreased compared with female offspring rats (Huang et al., 2017). However, in the human study, it was reported that the mean rate of 5-HT synthesis was higher in normal males than in normal females (Nishizawa et al., 1997). Further study is required to verify whether expression levels of these genes are physiologically different or resulted from stress treatments. Our immunofluorescence staining results did not denied that 5-HT production was promoted in the DRN after KSS treatment. Serotonergic neurons in the DRN are reported to project to distinct brain areas, such as PFC and nucleus accumbens (Waselus et al., 2011). Given these investigations, we postulate one hypothesis that KSS administration induces *Tph1* and *Tph2* gene expression in the DRN, and consequently reduces ABB. Further research is needed to examine the relation between 5-HT increase in the DRN and other brain areas regulating aggressive behaviors. It would be helpful to distinguish newly activated serotonergic neuron, for example, by coimmunostaining with anti-5-HT antibody and anti-c-Fos antibody.

As many as seven subfamilies of 5-HT receptors exist, each of which induces distinct responses (Żmudzka et al., 2018). For example, 5-HT_{2C} receptors in the bed nucleus of the stria terminalis have been implicated in fear-promoting action of mice (Marcinkiewcz et al., 2016). Additionally, a 5-HT_4 receptor agonist has been shown to acutely reduce immobility time of rats in a forced swim test (Lucas et al., 2007). However, based on the results of our experiment, in which we used a 5-HT_{1A} receptor antagonist, we propose that the reduction of ABB was achieved through activation of 5-HT_{1A} receptors. Although the exact mechanism underlying the induced selective action on 5-HT_{1A} receptors remains to be determined, a previous study reported that shogaol (composed of ginger) acts as a partial agonist on 5-HT_{1A} receptors (Nievergelt et al., 2010). Further study will be required to ascertain the concerted mechanism by which KSS components selectively act on 5-HT_{1A} receptors. The activity of 5-HT_{1A} receptors usually results in reduction of neuronal activity (Huang et al., 2017). However, the 5-HT_{1A} receptors have dual role: a first role as postsynaptic receptors to reduce different neuronal cells and a second role as autoreceptors to regulate serotonergic cell activity. Indeed, we found that *c-fos* gene expression is decreased in the DRN of both male and female mice that were administered with KSS, suggesting that 5-HT_{1A} autoreceptors in the DRN were activated in both male and female mice that were administered with KSS. It was also found that *c-fos* gene expression in the PFC of male mice that were administered with KSS was increased,

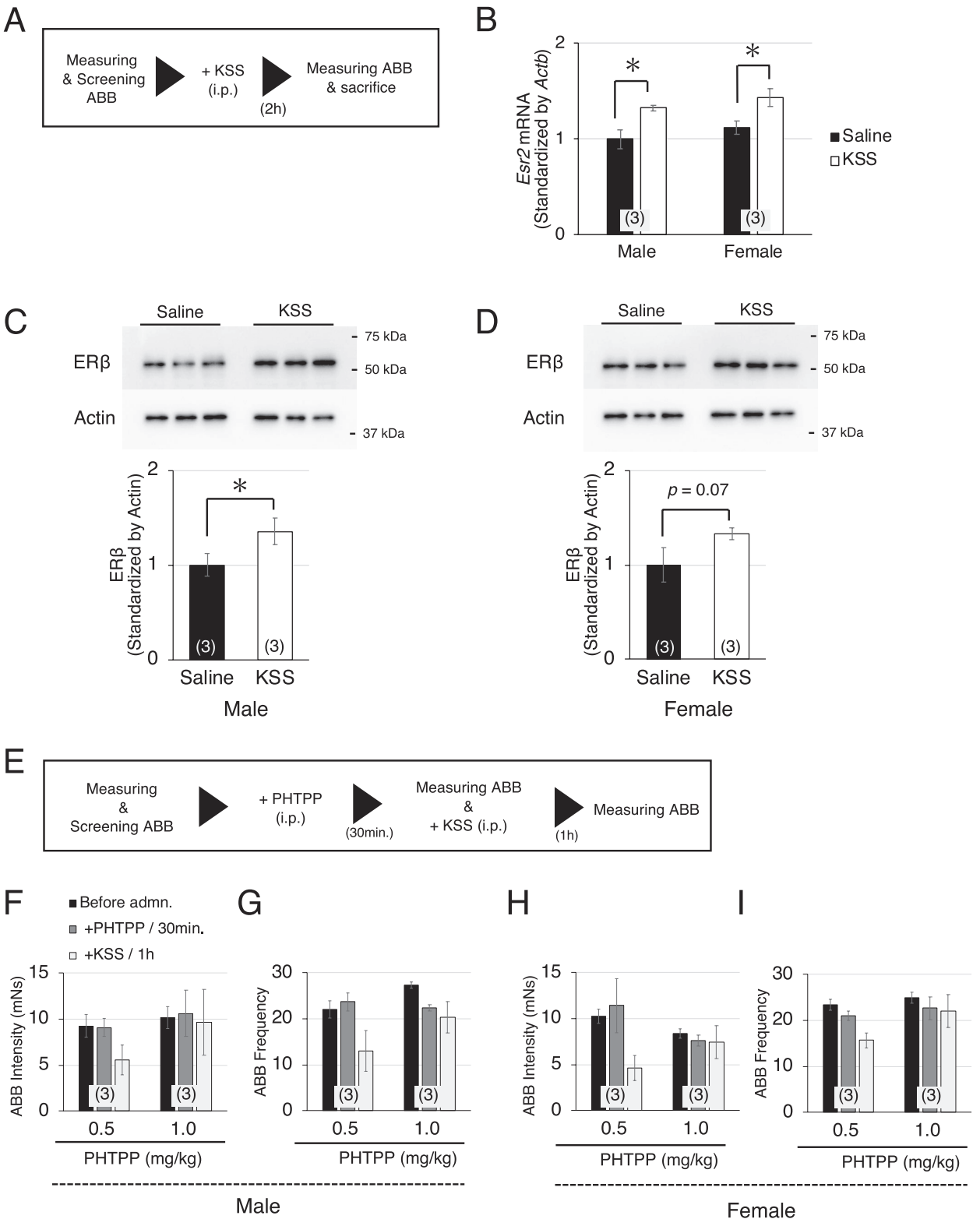

Fig. 9. The effect of Kamishoyosan (KSS) administration on *Esr2* mRNA expression and ERβ protein expression in the DRN of mouse brains. (A) Scheme of the experimental procedure for expression analysis. Brain tissue fractions from the DRN were resected from mice administered with KSS (100 mg/kg) or saline at 2 h after administration. (B) Expression levels of *Esr2 (ERβ)* according to qRT-PCR; data were normalized to *Actb* mRNA expression and shown as relative values compared with expression levels in male mice administered with saline ($n = 3$ animals in each group). Error bars represent standard deviations. * $p < 0.05$ (two-way ANOVA followed by Tukey's HSD test). (C, D) Expression levels of ERβ according to western blot; data were normalized to Actin protein expression and shown as relative values compared with expression levels in male (C) or female (D) mice administrated with saline (n = 3 in each group). Error bars represent standard deviations. * $p < 0.05$ (Student's t test). (E) Scheme of the experiment procedure for inhibitor analysis. (F–I) The biting behaviors of male (F, G) and female (H, I) mice. The intensity and frequency of biting behaviors in these measurement sessions were respectively averaged within each experimental group; thus, data represent means ± standard errors. Numbers in brackets represent numbers of mice used in each experimental group. Data were analyzed using one-way ANOVA and statistical significance at $p < 0.05$ was not detected.

while *c-fos* gene expression in the PFC of female mice that were administered with KSS was decreased. In the previous study, it was reported that male rats show higher performance in context-mediated renewal test and Fos-positive neurons in the PFC compared with females and that the males' performance was lowered by silencing the neuroactivation in the PFC, suggesting that a certain sex-specific physiological machinery activates or suppresses Fos induction in the PFC (Anderson and Petrovich, 2018). Further study is needed to elucidate how KSS works on distinct 5-HT_{1A} receptors and related neuronal circuits.

As we have discussed, our results suggest that activation of serotonergic neurons in the DRN may be involved in the efficacy of KSS against ABB. On the other hand, our results also indicate that KSS reduces ABB in male mice through combinatory action on the GABAergic and serotonergic systems. In addition, it was previously suggested that neural crosstalk between the GABAergic and serotonergic neuron (Huang et al., 2017). Thus, it is possible that KSS reduces male mouse ABB partly by sedative action that occurs via the GABAergic system. Additional studies are required to elucidate the synergistical action mechanism of KSS and sex differences in the neural circuits that govern pathological aggression.

We found that KSS-mediated reduction in mouse ABB was inhibited by preadministration of ER antagonists, suggesting that potential roles of ERα, ERβ, and GPR30 on the ABB-reducing action of KSS. It has been reported that ERα and ERβ have different distributions in the mouse brain (Mitra et al., 2003; Sheng et al., 2004; VanderHorst et al., 2005). In the present study, we focused the expression of ERs in the DRN, since increases of *Tph1* and *Tph2* mRNA expression were observed in the DRN, which is a major source of brain serotonin. Indeed, we found that *Tph2* mRNA induction in the DRN of male mice was abolished by preadministration of ER antagonists. It was previously reported that around the midbrain, ERβ is predominantly distributed in the DRN, whereas ERα is predominantly distributed in the periaqueductal gray (Mitra et al., 2003; Nomura et al., 2005). ER, when bound to agonists, dimerize and interact with regulatory DNA sequences to modulate gene transcription (Klinge, 2001; Powell and Xu, 2008; Yaşar et al., 2017). Interestingly, the colocalization of ERβ with TPH in the DRN has been reported as ~ 96% (Nomura et al., 2005; Sheng et al., 2004). In addition, Gundlah et al. (2005) reported that the *Tph* mRNA signal detected by *in situ* hybridization in ERβ-deficient mice was lower than that detected in wild-type mice. We found that *Esr2* mRNA expression and ERβ protein expression are increased by about 30% in the DRN of male and female mice. We also found that an ERβ-selective antagonist inhibits ABB-reducing action of KSS, supporting the notion that the action of KSS on ERβ might be involved in *Tph* induction and reduced aggressiveness. However, it has been reported that ERs are distributed not only in the DRN but also in a variety of brain regions and other neurotransmitter systems, whose contribution to the ABB-reducing effect of KSS have yet to be examined (Cui et al., 2013; Isgor et al., 2003).

Kumagai et al. (2005) showed some activity of KSS on ERs using a reporter assay; therefore, we compared the action of 17α- and 17β-E2 on ABB. It has also been reported that KSS and liquiritigenin, the estrogenic compound from the root of *Glycyrrhizae uralensis*, act agonistically on ERβ (Mersereau et al., 2009; Watanabe et al., 2006). Taken together, the results of the present and previous studies suggest that a certain ingredient of KSS might act on ERβ in the DRN neurons to facilitate *Tph1* gene expression and 5-HT production. A 3D-HPLC chart of KSS, which has previously been described in Wang et al. (2018), would be useful for determining the active pharmaceutical ingredients of KSS in relation to reduced ABB. Interestingly, a trace amount of E2 was capable of reducing ABB, although it can be hypothesized that mice had sufficient flavonoid levels, since mice were fed with an ordinary diet, not depleted in phytoestrogens such as genistein and daidzein, ingredients of soybeans (Lian et al., 2001; Thigpen et al., 1999). Further study is required to explore and characterize the estrogen-like ingredients in KSS.

Finally, our results indicate that the action on 5-HT_{1A} receptor is associated with the reduction of ABB and the action of KSS on ERs might stimulate 5-HT production. However, since KSS is composed of 10 herbs, work remains to be done to elucidate the action of each herb and how their combination concertedly exert the complex action of KSS.

4. Conclusion

KSS is widely used as a traditional herbal formula in Japan to improve psychiatric symptoms, such as general malaise, in menopausal women. However, limited ethological validation of KSS action has been reported until recently. In the present study, we showed that KSS effectively reduces ABB in isolation-reared male and female mice presumably through stimulation of 5-HT production in the DRN. In male mice, the combinatory effect of KSS on 5-HT_{1A} receptors and $GABA_A$ receptors seems to be involved in reducing ABB. In female mice, however, the effect of KSS on 5-HT_{1A} receptors seems to be the primary element in ABB reduction. Our findings also suggest that increased ERs are associated with the action of KSS. Further research building on the results provided here should focus on the individual components of KSS; such studies will provide a better understanding of the synergistic effect of KSS and its ability to improve psychiatric symptoms.

5. Materials and methods

5.1. Animals

All experiments were performed using ddY-strain mice, which were purchased from Japan SLC (Slc: ddY; Japan SLC, Inc., Shizuoka, Japan). Normal male and female mice were housed together for mating to obtain offspring. For the isolation-rearing experiments, male and female pups were separated from the dam at 3-weeks-old and then isolation-reared in individual cages (16 × 23 × 12 cm) until 11 weeks after birth. For the group-housing experiments, pups were separated from the dam at 3 weeks and housed in groups of 2–3 per cage (32 × 23 × 12 cm) until 11 weeks after birth. Measurement of ABB was performed within the period during which they had received 6–8 weeks of isolation rearing (i.e., postnatal week 9–11; see Supplementary Fig. 1). To assess the effects of a single oral administration of KSS, it was administered to isolation-reared mice using a plastic sonde (Fuchigami Kikai, Kyoto, Japan). For i.p. administration of drugs, each mouse was placed on wire netting, picked up by its tail, and the drug was intraperitoneally administrated using a Terumo 29 × 1/2″ gauge syringe (Terumo Co., Tokyo, Japan), as previously described (Kuchiiwa and Kuchiiwa, 2014, 2016). Monitoring of vaginal smear images was performed following the method of McLean et al. (2012). All animals were housed under controlled conditions (temperature: 22 °C ± 2 °C; lighting: lights on at 7:00, lights off at 19:00), with food and water administered *ad libitum*. Cage exchange was performed every 10 days. All animal procedures were approved by the Committee of Animal Experimentation, Kagoshima University, and performed in accordance with the guidelines of the Japanese Pharmacological Society.

5.2. Drugs and reagents

KSS (TJ-24; Tsumura Co. Ltd., Tokyo, Japan) was purchased as a freeze-dried powdered extract. A daily dose of powder (7.5 g) of this medicine contains 4.0 g of KSS extract obtained from the following 10 herbs: *Bupleurum falcatum* (3.0 g), *Paeonia lactiflora* (3.0 g), *Atractylodes lancea* (3.0 g), *Angelica acutiloba* (3.0 g), *Paria cocos* (3.0 g), *Gardenia jasminoides* (2.0 g), *Paeonia suffruticosa* (2.0 g), *Glycyrrhizae uralensis* (1.5 g), *Zingiber officinale* (1.0 g), and *Menthae arvensis* (1.0 g) (Yamada and Kanba, 2007). Magnesium stearate and lactose hydrate are added as inactive ingredients for granulation by the manufacturer. At each experimental day, KSS suspension was prepared by vortex. The suspension of KSS was intraperitoneally injected to the subject mouse at the experimental day within postnatal week 9–11 at 100 mg/kg or orally

administered at 100, 200, or 400 mg/kg.

N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide (WAY-100635) maleate (ab120550; Abcam, Cambridge, UK) and [*S*-(R*,*S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-*e*]-1,2-benzodioxol-6-yl)5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dimethyl-1,3-dioxolo[4,5-*g*]isoquinolinium iodide ((-)-bicuculline) methiodide (ab120108; Abcam) were dissolved in distilled water and intraperitoneally administered to each mouse (0.5 mg/kg and 1 mg/kg, respectively). The doses of WAY-100635 and (-)-bicuculline were determined according to the previous studies (Guo et al., 2019a; Harada et al., 2018; Kanno et al., 2009; Magen et al., 2010; Mizowaki et al., 2001). Tamoxifen (2-[4-[(1Z)-1,2-diphenyl-1-buten-1-yl]phenoxy]-N,N-dimethyl-ethanamine, item no. 13258; Cayman Chemical Company, MI, USA) and PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a] pyrimidin-3-yl]phenol, Selleck Biotech, Tokyo, Japan) were suspended in corn oil containing 10% ethanol. ICI-182,780 ((7*R*,9*S*,13*S*,14*S*,17*S*)-7-(9-(4,4,5,5,5-Pentafluoropentylsulfinyl)nonyl)-7,8,9,11,12,13,14,15,16,17-decahydro-13-methyl-6*H*-cyclopenta[*a*] phenanthrene-3,17-diol, ab120131; Abcam) and G-15 ((3aS,4R,9bR)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinoline, item no. 14673; Cayman Chemical Company) were respectively suspended in corn oil containing 5% ethanol. 17α- and 17β-E2 were purchased from Nacalai Tesque Inc. (Kyoto, Japan; code: 145–48 and 145-41, respectively) and dissolved in corn oil containing 1% ethanol. The doses of tamoxifen, PHTPP, ICI-182780, and G-15 were determined according to previous studies using mice (Alfinito et al., 2008; Kim et al., 2010; Lai et al., 2017; Li et al., 2016; Liu et al., 2010; Shooshtari et al., 2021; Zhao et al., 2017). The doses of 17α- and 17β-E2 were determined based on assumption of the previous study (Kumagai et al., 2005).

5.3. ABB measurement

The intensity and frequency of ABB toward an inanimate object was measured using an ARM device (Muromachi Kikai Co. Ltd., Tokyo, Japan), as previously described (Kuchiiwa and Kuchiiwa, 2014, 2016). In brief, the subject mouse is placed in the cylinder on the top of ARM and measured ABB (see Supplementary Fig. 1). Measurement of ABB is consisted of two sessions; first irritation session and second measurement session. Metal rods with a dome-shape head are automatically controlled to make an up-and-down motion 30 times to touch the hind limb of the subject mouse in the first session and to propose the rods in front of the subject mouse in the second session. The load sensor connected to the rods measures ABB intensity (average intensity of one biting) and ABB frequency (total number of bitings within 30 presentations of the rods). ABB intensity is expressed in numerical values as an integral of biting force within one-second presentation of the rods (milliNewton × second: mNs).

5.4. Quantitative RT-PCR

To surgically resect brain tissue, mice were first deeply anesthetized with pentobarbital (100 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and then transcardially perfused with phosphate-buffered saline (PBS). To obtain the tissue of the DRN, we referred to the Franklin and Paxinos' mouse brain atlas (Franklin and Paxinos, 2007). Briefly, the brain was first coronally sliced on ice at the inferior colliculi. After that, a triangle tissue fraction, extending about 0.8–1 mm from the aqueduct to whitish area and about 0.5 mm width from the aqueduct to each bilateral whitish area was resected. This triangle tissue fraction was used as DRN. Each fraction was homogenized in Isogen (Nippongene, Tokyo, Japan) and stored at −80 °C until total RNA was prepared; at this point, the fractions of three mice per group were mixed. After using a standard extraction procedure using Isogen, following DNase I (QIAGEN) treatment, and purification using NucleoSpin RNA Clean-up kit (Macherey Nagel GmbH & Co. KG), 600 ng of total RNA was used for reverse transcription with ReverTra Ace (Toyobo, Osaka, Japan). First-strand cDNA was stored at −20 °C until use. Relative gene expression was analyzed via the relative standard curve method with THUNDERBIRD SYBR qPCR Mix (Toyobo) on an ABI 7300 real-time PCR system (Applied Biosystems, Thermo, MA, USA). The forward and reverse primer sequences used were 5′-ACA TGC CAA AGT CAA GCC CT-3′ and CCG CAA CTC ATT CAT GGC AC for *Tph1* (ENSMUST00000049298); 5′-AGA TGC TCT CAC CGA GTC CT-3′ and 5′-GCA AGC ATG AGT CGG GTA GA-3′ for *Tph2* (ENSMUST00000006949); 5′-CTG TCC AGC CAC GAA TCA GT-3′ and 5′-GGC TTT GTT CAG GCA ATG CA-3′ for *Esr2* (ENSMUST00000076634); 5′- TGT TCT CGG GTT TCA ACG CC-3′ and 5′-CTG GTG GAG ATG GCT GTC AC-3′ for *c-fos* (ENSMUST00000021674); 5′-GAG GCC CAG AGC AAG AGA GG-3′ and 5′-GCT ACG TAC ATG GCT GGG GT-3′ for *Actb* (ENSMUST00000100497). The primer sequence was designed using Primer3Plus software (Untergasser et al., 2012).

5.5. Immunohistochemistry

Mice were deeply anesthetized with pentobarbital (100 mg/kg) and transcardially perfused with PBS (pH 7.4) followed by 4% formaldehyde. After post fixation, brains were equilibrated in 30% sucrose overnight at 4 °C. Brain tissues were frozen in O.C.T. compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) at −80 °C overnight, after which they were coronally sectioned to a thickness of 40 μm on a freezing microtome (CryoStar NX70 Cryostat; Thermo) and stored in PBS supplemented with 0.1% sodium azide at 4 °C until use.

For immunostaining, sections were blocked with 1% BSA in PBS supplemented with 0.2% Triton X-100 at room temperature for 1 h. Sections were then incubated with anti-5-HT antibody (S5545; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany; 1:1,000) as the primary antibody at room temperature overnight. The sections were subsequently incubated with Alexa488-conjugated anti-rabbit IgG antibody (A-11008; Thermo; 1:1,000) as the secondary antibody at room temperature for 3 h. Each section was counterstained with 4′6-diamidino-2-phenylindole (i.e., DAPI). Images were captured on a confocal microscope system (Leica TCS SP8; Leica Microsystems, Tokyo, Japan).

For measuring the mean immunofluorescence intensity of DRN in each mouse, three sections (approximately around bregma −4.72) were used per an individual by referring to the standard mouse brain atlas (Franklin and Paxinos, 2007). Immunofluorescence intensity of each image was quantified by using ImageJ (NIH).

5.6. Immunoblot analysis

For immunoblot analysis, each fraction was mixed in cell lysis buffer (containing 50 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM NaF, and 1 mM sodium orthovanadate) supplemented with protease inhibitor cocktail (Nacalai Tesque); after centrifugation of this mixture, the supernatant was collected as protein extract. Following reduction with 100 mM dithiothreitol under thermal denaturing conditions (95 °C for 10 min.), 10 μg of each protein extract was electrophoresed on polyacrylamide gel (10% acrylamide, 29:1) and blotted on a polyvinylidene difluoride (PVDF) membrane (Cytiva, UK). Blocking was performed with 2% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.1% Tween-20 for 30 min. Anti-β-actin antibody (no. 4970; Cell Signaling Technology, MA, USA; 3,000) and anti-ERβ antibody (PA1-310B; Thermo; 1:1,000) were used as primary antibodies, whereas anti-Rabbit IgG, HRP-linked antibody (no. 7074; Cell Signaling Technology; 3,000) was used as the secondary antibody. After activation with ImmunoStar Zeta (Fujifilm-Wako, Osaka, Japan), chemical luminescence was captured on a ChemiDoc XRS + system (BioRad, CA, USA).

5.7. Statistical analysis

Tukey's HSD test or Tukey–Kramer test after one-way or two-way ANOVA was used for analyses. Student's *t* test was used for analysis of ABB after 17α- or 17β-E2 treatment. Significance was set at $p < 0.05$. Statistical analyses were performed using StatPlus software (AnalystSoft Inc., CA, USA).

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CRediT authorship contribution statement

Kento Igarashi: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft. **Toshiko Kuchiiwa:** Conceptualization, Writing - review & editing. **Satoshi Kuchiiwa:** Conceptualization, Writing - review & editing. **Haruki Iwai:** Methodology, Writing - review & editing. **Kazuo Tomita:** Data curation, Writing - review & editing. **Tomoaki Sato:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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