Behavioural changes in young ovariectomized mice via GPR30-dependent serotonergic nervous system

（ GPR30 によるセロトニン神経系の調節を介した若年期卵巣摘出モデルマウスの行動変化 ）

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RESEARCH REPORT

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Behavioural changes in young ovariectomized mice via GPR30-dependent serotonergic nervous system

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Abstract

Fluctuations in estradiol levels at each stage of life in women are considered one of the causes of mental diseases through their effects on the central nervous system. During menopause, a decrease in estradiol levels has been reported to affect the serotonin nervous system and induce depression-like and anxiety symptoms. However, the regulation of brain and behaviour during childhood and adolescence is poorly understood. Moreover, the role of oestrogen receptors α and β in the regulation of the serotonergic nervous system has been reported, but little is known about the involvement of G protein-coupled receptor 30. Therefore, in this study, we used an ovariectomized childhood mouse model to analyse behaviour and investigate the effects on the serotonin nervous system. We showed that ovariectomy surgery at 4 weeks of age, which is the weaning period, induced a decrease in spontaneous locomotor activity during the active period and a preference for novel mice over familiar mice in the three-chamber social test at 10 weeks of age. In addition, the administration of G-1, a protein-coupled receptor 30 agonist, to ovariectomized mice suppressed spontaneous locomotor activity and the preference for novel mice. Furthermore, we demonstrated that childhood ovariectomy induces increased tryptophan hydroxylase gene expression in the raphe nucleus and increased serotonin release in the amygdaloid nucleus, and administration of G-1

Abbreviations: ERα, oestrogen α receptor; ERβ, oestrogen β receptor; GPR30, G protein-coupled receptor 30; ICR, Institute of Cancer Research; OVX, ovariectomized; RT-qPCR, Reverse Transcription quantitative Polymerase Chain Reaction; SERT, serotonin transporter; TPH, tryptophan hydroxylase; 5-HT_{1A}R, 5-hydroxytryptamine $_{1A}$ receptor.

ameliorated these effects. Our study suggests that G protein-coupled receptor 30-mediated regulation of serotonin synthesis is involved in changes in activity and social-cognitive behaviour due to decreased estradiol levels during childhood.

KEYWORDS
G protein-coupled receptor 30, OVX, serotonin, social behaviour, tryptophan hydroxylase

1 | INTRODUCTION

Ovarian hormones, such as estradiol, are known to be involved not only in reproductive development but also in memory and emotional regulation through their effects on the central nervous system. Fluctuations in estradiol levels at each stage of life in women are considered one of the causes of various disorders such as depression and anxiety (Del Río et al., 2018). Estradiol is neuroprotective and modulates neurotransmitter release (Barreto et al., 2021; Borrow & Cameron, 2014). For example, a decrease in estradiol during menopause induces a decrease in dopamine, serotonin and noradrenaline levels, leading to depression-like and anxiety symptoms (Eid et al., 2020; Heydarpour et al., 2013; Izumo et al., 2012). In addition, estradiol is thought to be involved in brain differentiation and behaviour regulation from childhood to adolescence (McCarthy, 2008). Estradiol has also been implicated in attention deficit, hyperactivity and memory impairment in developmental disorders and Turner syndrome (Filice et al., 2018; Wolstencroft et al., 2022). However, there is a limited understanding of how estradiol modulates emotional behaviour from childhood through adolescence.

The regulation of behaviours by estradiol has been reported to involve the serotonergic nervous system. Estradiol signalling pathways include the classical pathway via the oestrogen α and β receptors (ERα and ERβ, respectively) and the G protein-coupled receptor 30 (GPR30)-mediated pathway. ERα and ERβ, which are primarily nuclear receptors, have been proposed to regulate enzyme levels, receptors and transporters in serotonergic neurons through interactions with transcription factors, such as C/EBPβ, NF-κB and AP-1. It has been reported that ERβ mediates the estradiol-induced increase in tryptophan hydroxylase (TPH) activity of serotonin synthase (Hiroi & Handa, 2013). Previous studies have revealed that ERα and ERβ are co-localized with the serotonin transporter (SERT) in serotonin neurons of the dorsal raphe nucleus, and estradiol treatment in the ovariectomized (OVX) mouse model downregulates SERT mRNA levels in the midbrain raphe nucleus in rodents (Bethea et al., 2002; Zhou et al., 2002). Furthermore, ERα and ERβ have been proposed to regulate the 5-HT_{1A} receptor (5-HT_{1A}R; Gundlah et al., 2001; Wissink et al., 2001). However, little is known about the effects of GPR30 on the serotonergic system. GPR30 has been reported to be involved in spatial memory, social cognition and anxiety (Hadjimarkou & Vasudevan, 2018; Vajaria & Vasudevan, 2018), and the serotonergic nervous system has been proposed to play an important role in social and anxiety behaviours (Bacqué-Cazenave et al., 2020). Therefore, it is important to understand the mechanism of serotonin nervous system regulation by GPR30.

This study focused on the role of estradiol during adolescence, which is important for brain development and the acquisition of social behaviour and aimed to determine its effects on behaviour and the regulation of the serotonergic nervous system via GPR30. To address this issue, we performed various behavioural experiments in adolescents using OVX mouse models during weaning. In addition, we examined the expression of genes involved in the nervous system and serotonin release in the brain.

2 | MATERIAL AND METHODS

2.1 | Animals and operating

Pregnant ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and female mice weaned at 4 weeks of age were used in this study. The breeding environment was set to room temperature (24 ± 1°C), ambient humidity (55 ± 5%) and 12-h light/dark cycles (light period: 07:00–19:00; dark period: 19:00–07:00). Food (Labo MR stock; Nosan Corporation, Kanagawa, Japan) and water were provided ad libitum. Four-week-old mice were randomly allocated to one of three groups and given surgery; one group received a sham surgery (Sham), whereas the other two groups underwent OVX, as previously described (Izumo et al., 2012). After surgery, mice were kept in separate cages from the parent mice, with 4–6 mice per cage per group. Once a day from 1 week after surgery until 12 weeks of age at

the end of the experiment, one of the OVX groups was injected subcutaneously with 330 μg/kg of G-1 (Sigma-Aldrich, St. Louis, MO, USA). The dose of G-1 was calculated according to the study of Anchan et al. (2014). The sham group and other OVX group were injected with the solvent (10% dimethyl Sulfoxide [FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan], 5% Tween 80 [Tokyo Chemical Industry Co., Ltd., Tokyo, Japan] and 85% saline) as the vehicle. The present study was approved by the Animal Experiment Committee of Yokohama University of Pharmacy (approval number: 2022-003), and care was taken to ensure the welfare of the laboratory animals.

2.2 | Behaviour

Behavioural tests were performed at 10–12 weeks of age using 15–20 mice. The open field test, marble-burying behaviour test and three-chamber social test were performed from 11:00 to 16:00 during the light phase. The mice were acclimatized to the test chamber for 1 h before testing. Spontaneous locomotor activity and open field tests were performed in all mice, and mice were only performed in either marble-burying behaviour test or three-chamber social test to account for the load of the behavioural test. Behavioural tests were administered in the following order: spontaneous locomotor activity, open field test, marble-burying behaviour test or three-chamber social test; administration was done after each behavioural test (15:00–18:00).

2.3 | Spontaneous locomotor activity

The mice were placed in individual cages using a spontaneous activity-measuring device (NS-AS01; Neuroscience, Tokyo, Japan), and the amount of activity per hour was measured using an infrared sensor. The measurements were performed for 24 h.

2.4 | Open field test

Behaviour in a novel environment was measured using an open field apparatus (50 cm × 50 cm × 50 cm). Each mouse was placed in the centre of the open field apparatus and recorded for 15 min, after which the device was wiped clean with 70% ethanol. The central zone was defined as the central area (25 cm × 25 cm), and the distance travelled, time spent and head-stretching behaviour were calculated using viewer^2 version2.2.0.17 (Biobserve GmbH, Bonn, Germany).

2.5 | Marble-burying behaviour test

The marble-burying behaviour test was performed according to the method by Himanshu (Himanshu et al., 2020). Briefly, an unscented bedding material was added to a standard polycarbonate cage (35 cm × 35 cm × 27 cm) to a depth of 5 cm. After 30 min of habituation, 20 marbles were evenly placed throughout the bedding, and the mice were left for the 30-min test period. After 30 min, each mouse was returned to its home cage, and all marbles buried 2/3 area or more were counted, but those buried less than 2/3 area were not.

2.6 | Three-chamber social test

Sociability and social novelty preference were assessed according to the method proposed by Kaidanovich-Beilin (Kaidanovich-Beilin et al., 2011). Briefly, the test was divided into three sessions: the habituation period, session 1 and session 2. Following the 5-min habituation period, the mice were placed back into the centre, and an unfamiliar female ICR mouse was placed in one of the two cups (session 1). After session 1, another unfamiliar female ICR mouse was placed in an empty cup. The duration spent in each chamber was measured for 10 min in sessions 1 and 2.

2.7 | Microdialysis

The day after all behavioural tests were completed, mice were randomly assigned, one for microdialysis and the other for reverse transcription quantitative polymerase chain reaction (RT-qPCR). Microdialysis was performed as previously reported, with minor modifications (Funada & Hara, 2001; Izumo et al., 2012). Briefly, measurements were taken 48 h after surgery, during which time the mice were allowed to recover from surgery, wake up and move freely, one per cage. The mice were anesthetized with 3% isoflurane in room air using a small animal anesthetizer (MK-AT210D, Muromachi Kikai Co., Ltd., Tokyo, Japan) and placed in a stereotaxic apparatus. A guide cannula was implanted just above the prefrontal cortex (A: +1.9 mm, L: +.5 mm, V: .8 mm relative to bregma) or the amygdaloid nucleus (A: −.94 mm, L: +2.1 mm, V: −4.0 mm relative to bregma). Probes were perfused with Ringer's solution (147-mM NaCl, 2.4-mM $CaCl_2$ and 4.0-mM KCl, pH 7.0) at a flow rate of 1 μL/min using a microperfusion pump (HTEC-500, Eicom, Kyoto, Japan). One hour after the insertion of the microdialysis probes, when neurotransmitter release stabilized, 20-min

fractions were collected. The amounts of serotonin and dopamine in the dialysate samples were quantified using high-pressure liquid chromatography and electrochemical detection (HTEC-500, Eicom).

2.8 | RT-qPCR

The brain tissue was removed rapidly after euthanasia by cervical dislocation the day after all behavioural tests were completed. To extract total RNA, Isogen (Nippon Gene, Tokyo, Japan) was added to the hippocampus, frontal lobe and midbrain to pons specimens extracted from each mouse, and the tissues were homogenized with POLYTRON PT 1300 D (Central Scientific Commerce, Tokyo, Japan). Chloroform (Nacalai Tesque Inc., Kyoto, Japan) was added to the homogenized samples, which were then centrifuged, and the supernatant was collected in a new tube. Isopropanol (Nacalai Tesque, Inc.) was added to the supernatant, and the precipitate was collected by centrifugation. The precipitate was then resuspended in sterile water, the RNA concentration was measured and cDNA was synthesized using the PrimeScript™ RT Master Mix (Perfect Real Time; Takara Bio, Shiga, Japan) protocol, with incubations for 15 min at 37°C and 5 s at 85°C. For real-time PCR, a Light Cycler 96 (F. Hoffmann-La Roche, Basel, Switzerland) was used with a primer pair for each marker (Table 1), and the TaqMan probe method was followed for 40–50 cycles, where each cycle consisted of 95°C for 10 min, 95°C for 10 s and 60°C for 20–30 s.

2.9 | Statistical analysis

Data were analysed using the Kolmogorov–Smirnov test for normal distribution and the Bartlett test for equivariance. After checking the normal distribution of data, for uterine weight, body weight, spontaneous locomotor activity, open field test, marble-burying behaviour test, microdialysis, and RT-qPCR, when the assumption of equivalence of variance holds, the Dunnett's test was performed; when there is a deviation from the equivalence assumption, the Games–Howell test was performed. The three-chamber social test was analysed by the paired *t* test. The statistical difference was considered significant when the *p* value was less than .05. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

TABLE 1 List of all primers used for RT-qPCR.

	Sequence	
Gene	**Forward**	**Reverse**
GAPDH	agcttgtcatcaacgggaag	tttgatgttagtggggtctcg
TPH	cacagttcagatcccctctaca	gaacgtggcctaggagttca
SERT	acctggacactccattccac	cctggagtccctttgactga
5-HT_{1A} receptor	caggcaggcatggatatgtt	ctccagggacgttgtggt

Abbreviations: 5-HT_{1A} receptor, *Mus musculus* 5-hydroxytryptamine (serotonin) 1A receptor; GAPDH, *Mus musculus* glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SERT, *Mus musculus* serotonin transporter; TPH, *Mus musculus* tryptophan hydroxylase.

3 | RESULTS

3.1 | GPR30 does not affect OVX-induced weight change or uterine weight

To confirm the involvement of GPR30 in body growth, the mice were weighed once per week. Figure 1a shows the weekly weight changes. OVX mice showed a significant increase in body weight from 6 weeks of age (2 weeks after surgery; $p < .05$; Figure 1a), and this increase continued until 10 weeks of age. Administration of G-1 to OVX mice had no effect on weight gain (Figure 1a).

Blood estradiol levels are associated with uterine weight. We measured the uterine weights after the study to confirm the decrease in estradiol levels following OVX. Figure 1b,c shows the uterine weight and a photograph of a representative uterus. The OVX group showed a significant reduction in uterine weight compared to the Sham group ($p < .01$; Figure 1b). The OVX + G-1 group showed a significant decrease compared to the Sham only ($p < .01$), indicating that GPR30 did not affect the uterus.

3.2 | G-1 inhibits the OVX-induced decrease in spontaneous locomotor activity

Figure 2a shows circadian rhythm and hourly spontaneous locomotor activity data. Circadian rhythms did not differ between the groups. Figure 2b,c shows the total spontaneous locomotor activity of the mice during the active (dark) and inactive (light) 12-h periods. There was no significant difference in spontaneous locomotor activity during the inactive phase (Figure 2c); however, during the active phase, spontaneous locomotor activity was significantly lower in the OVX group than in the Sham

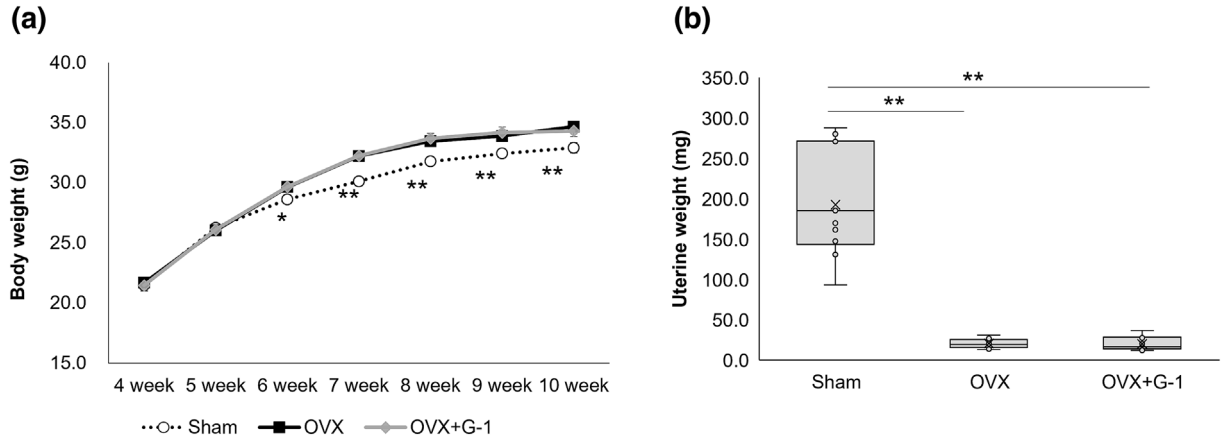

FIGURE 1 Ovariectomy (OVX) induced weight gain and decreased uterine weight in mice. Administration of G-1 had no effects with OVX induced body weight gain and uterine weight loss. (a) Changes in body weight of each group (**$p < .01$, *$p < .05$, Dunnett's test, comparison with OVX group). Line graph shows mean ± SE. (b) Uterine weight at 12 weeks of age of each group (**$p < .01$, Games–Howell test). Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

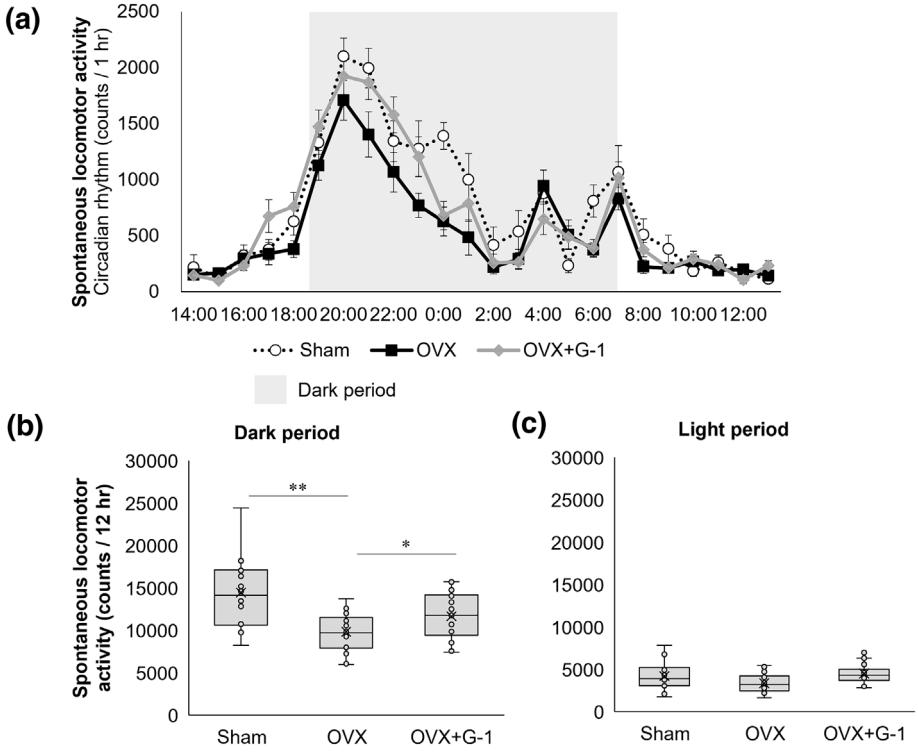

FIGURE 2 Ovariectomy (OVX) decreased spontaneous locomotor activity in dark period and which was suppressed by G-1 administration. (a) Circadian rhythm indicates spontaneous locomotor hourly activity. Line graph shows mean ± SE. (b,c) 12 h of spontaneous locomotor activity (**$p < .01$, *$p < .05$, Games–Howell test). The dark period indicates the hours from 19:00 to 7:00, and the light period indicates the hours from 7:00 to 19:00. Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

group ($p < .01$; Figure 2b). The OVX + G-1 group exhibited significant suppression of this OVX-induced decrease ($p < .05$; Figure 2b).

3.3 | G-1 does not affect behaviour change in open field test

We performed an open field test to evaluate the behaviour of mice in a novel environment. Figure 3a shows the total distance travelled, whereas Figure 3b,c shows the percentages of time and distance spent in the central area. There was no difference in the total distance travelled and the percentages of time spent in centre area between the groups (Figure 3a,b). On contrast, the percentage of distance travelled were significantly increased by OVX, but administration of G-1 had no effect on this increase ($p < .05$; Figure 3c). Figure 3d–f shows head-stretching behaviour and tail movements of the mice. Head bobs, head stretches and tail movements were

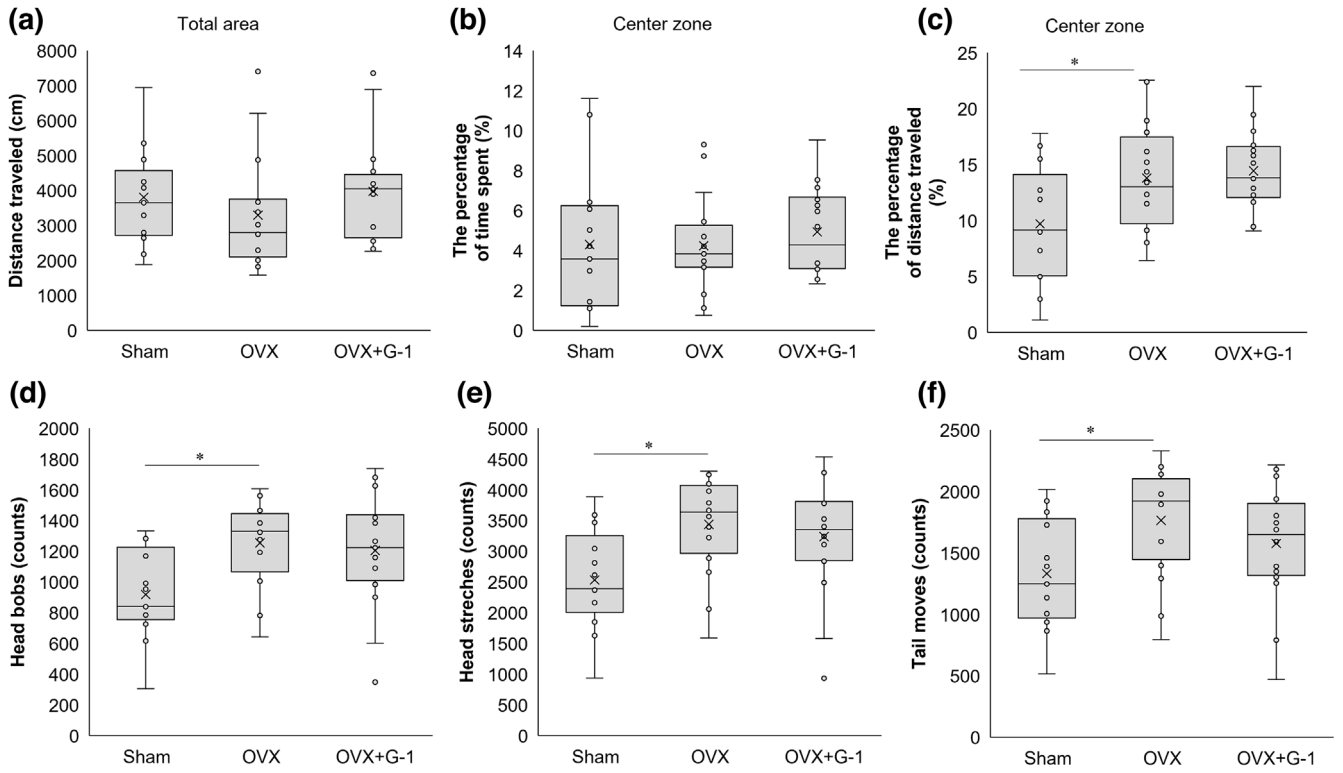

FIGURE 3 Results of open field test (OFT). (a) Distance travelled in total area, (b,c) percentage of time spent and distance travelled in the central area and (d–f) exploratory behaviour such as horizontal and vertical movement of the head (headbobs and headstreches) and tail (tailmoves). There were no significant differences between the groups in total distance travelled and percentage of time spent in the median (a,b). Ovariectomy (OVX) increased percentage of distance travelled and exploratory behaviour in OFT, but administration of G-1 had no significant effect (d–f) (*$p < .05$, Dunnett's test, comparison with OVX group). Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

significantly increased following OVX. However, the administration of G-1 had no effect on these increases (Figure 3d–f).

3.4 | G-1 does not affect obsessive-compulsive behaviour in a marble-burying behaviour test

Figure 4 shows the number of marble pieces buried out of 20 pieces. The behaviour of mice burying marbles is known as obsessive-compulsive behaviour. Compared with the Sham group, the OVX group tended to bury more marbles ($p = .089$; Figure 4). Administration of G-1 had no effect on OVX-induced compulsive behaviour (Figure 4).

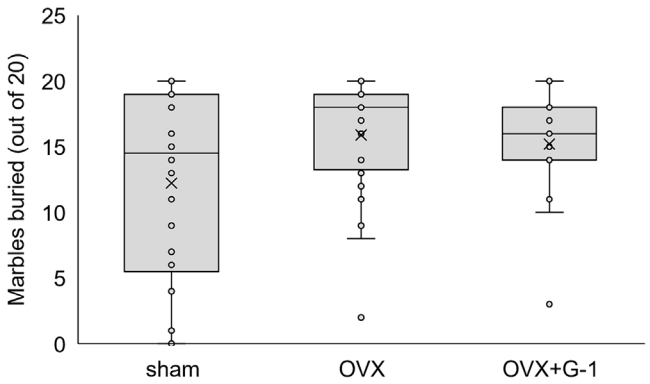

FIGURE 4 Results of marble-burying behaviour test. Data indicate the number of hidden marbles out of 20. Ovariectomy (OVX) showed an increasing trend of marbles buried, but administration of G-1 had no significant effect (*$p < .05$, Games–Howell test). Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

3.5 | G-1 inhibits OVX-induced changes in social novelty preference behaviour

Figure 5a,b shows the time spent in each chamber and the number of times each chamber was entered in session 1. Session 1 compared preferences for unfamiliar mice and the empty cups. In all groups, the time spent in the chambers with unfamiliar mice was significantly

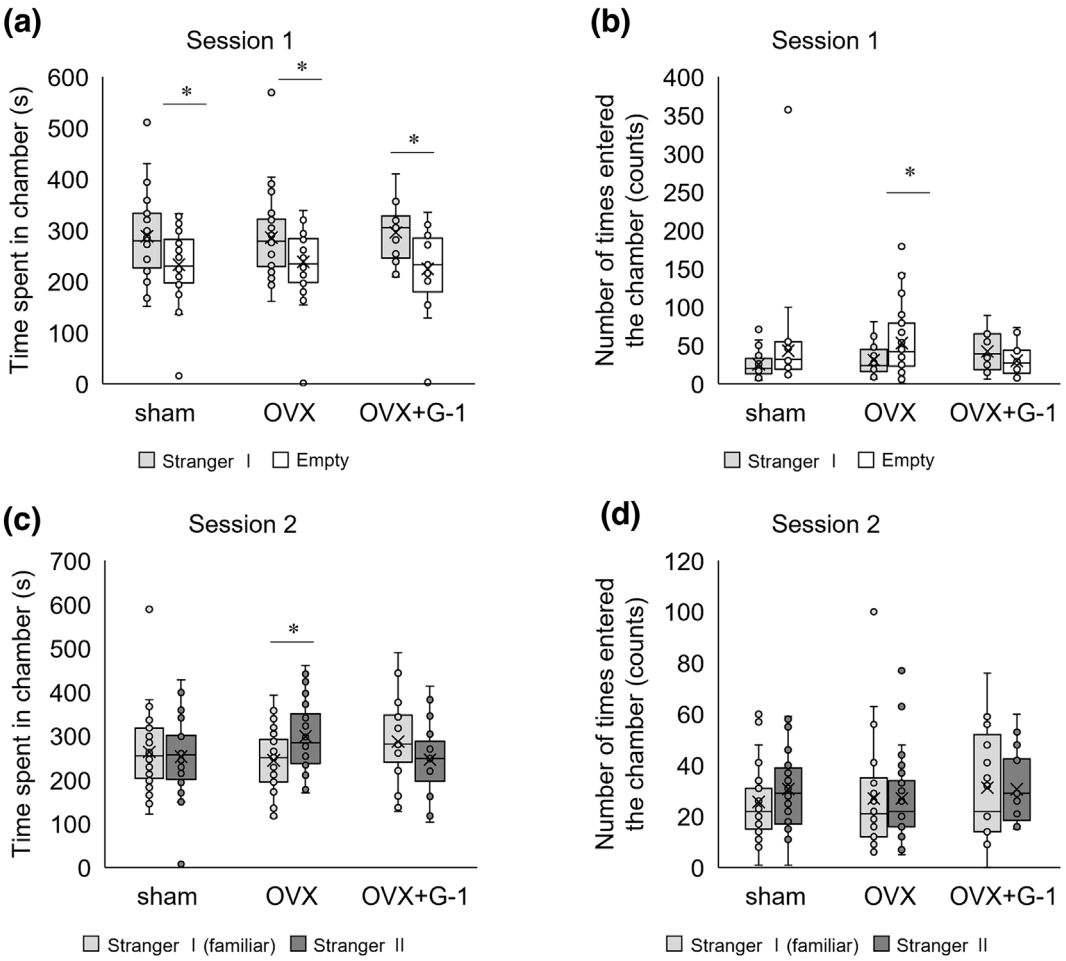

FIGURE 5 Results of three-chamber social test. (a,b) Session 1 shows the comparison of novel mice and novel objects preferences. (c,d) Session 2 shows the comparison of novel mice and familiar mice preferences. All groups showed a higher preference for novel mice compared to novel objects (a). Only ovariectomy (OVX) showed a higher preference for novel mice than for familiar mice (c) (*$p < .05$, paired t test). Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

longer than the time spent in the chambers with empty cups (Sham group; $t\ (38) = -2.4912$, $p < .05$; OVX group; $t\ (38) = -2.236$, $p < .05$, OVX + G-1 group; $t\ (16) = -2.2061$, $p < .05$; Figure 5a). Figure 5c,d shows the time spent in the chamber and the number of entries in session 2. Session 2 assessed preferences for unfamiliar and familiar mice (i.e., the mice contacted in session 1). OVX mice spent significantly more time in the chambers with unfamiliar mice than in the chambers with familiar mice ($t\ (38) = -2.2959$, $p < .05$; Figure 5c). In contrast, in the sham group, there was no significant difference in time spent in the chambers with unfamiliar or familiar mice ($t\ (38) = .40765$, $p = .686$; Figure 5c). The OVX + G-1 group, like the Sham group, also showed no preference for unfamiliar mice ($t\ (16) = .94833$, $p = .357$; Figure 5c).

3.6 | G-1 inhibits OVX-induced increases in serotonin release in the amygdaloid nucleus

We performed microdialysis to investigate the involvement of dopamine and serotonin release in the brain during OVX-induced behavioural changes. Figure 6 shows dopamine and serotonin release in the amygdaloid nucleus (Figure 6a,b) and prefrontal cortex (Figure 6c,d), respectively. There were no significant differences in dopamine release between the groups in either the amygdaloid or the prefrontal cortex (Figure 6a,c). In contrast, serotonin release in the OVX group was significantly elevated in the amygdaloid nucleus compared with that in the Sham group ($p < .05$; Figure 6b). Furthermore, the administration

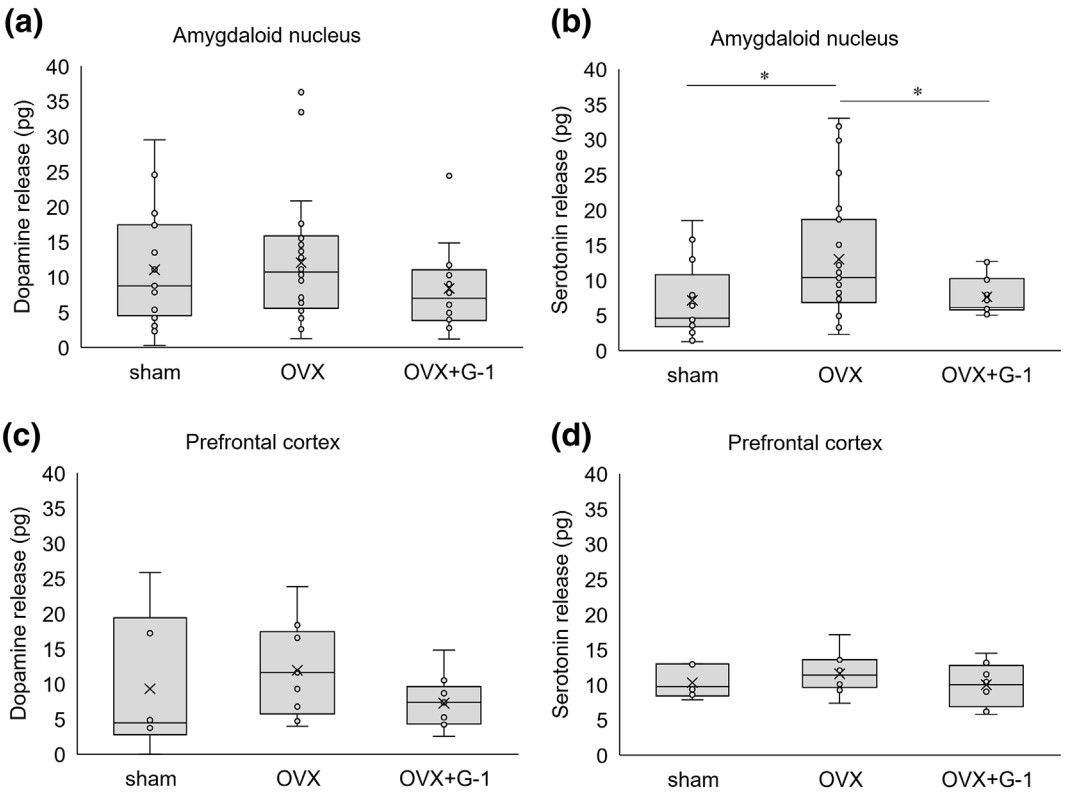

FIGURE 6 Dopamine and serotonin release in (a,b) the amygdaloid nucleus and (c,d) the prefrontal cortex by microdialysis. Data were collected and measured every 20 min for a total of 4 h. Dopamine release was not significantly different between groups in either the amygdaloid nucleus or the prefrontal cortex (a,c). Ovariectomy (OVX) increased serotonin release in the amygdaloid nucleus and which was suppressed by G-1 administration (b) (*$p < .05$, Games–Howell test). Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

of G-1 group significantly suppressed this OVX-induced increase.

3.7 | G-1 inhibits the increase in SERT gene expression in the midbrain and bridge

To determine the cause of the change serotonin release in the amygdala nucleus, the gene expression levels of serotonin-related molecules in the midbrain and bridge, hippocampus and prefrontal cortex were investigated. Figure 7 shows the gene expression levels of TPH, SERT and 5-HT_{1A}R in the midbrain and bridge (Figure 7a–c), hippocampus (Figure 7d–f) and prefrontal cortex (Figure 7g–i). Regarding the gene expression level of TPH in the midbrain and bridge, where the raphe nuclei are located, the OVX group showed a significant increase compared with the Sham group ($p < .01$; Figure 7a). The OVX + G-1 group exhibited a tendency towards a suppression in this OVX-induced increase ($p = .098$, Figure 7a). SERT gene expression levels in the midbrain and bridge were not significantly different between the Sham and OVX groups ($p < .05$; Figure 7b) but were significantly lower in the OVX + G-1 group than in the OVX group. In the hippocampus, the gene expression levels of TPH and SERT were not significantly different between the groups (Figure 7d,e). In contrast, the gene expression levels of 5-HT_{1A}R in the OVX group were significantly higher than those in the Sham group in hippocampus ($p < .05$; Figure 7f); however, G-1 administration did not affect this OVX-induced increase. In the prefrontal cortex, although the expression of 5-HT_{1a}R was increased by OVX, no significant differences were observed in the expression of TPH and SERT (Figure 7g–i).

4 | DISCUSSION

It has been proposed that estradiol plays an important role in brain development and in the acquisition of social behaviour during childhood (Yoest et al., 2023; Zwaan et al., 2022). Neurotransmitters, such as dopamine and serotonin, are involved in the regulation of social behaviour and anxiety-related behaviour, and estradiol plays a significant role in controlling the release of these neurotransmitters (Del Río et al., 2018; Hernández-Hernández

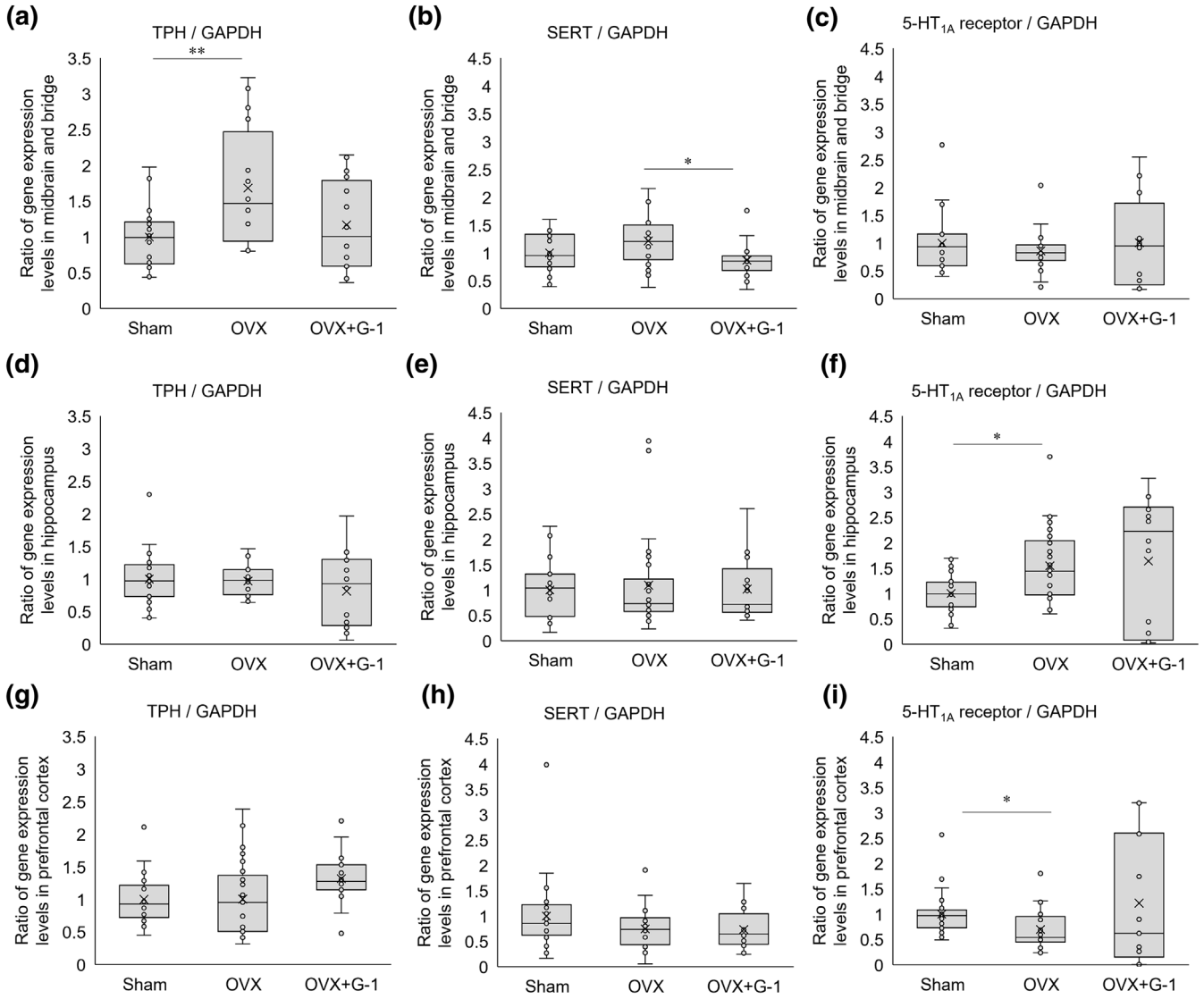

FIGURE 7 Expression levels of genes related to the serotonin nervous system in (a–c) the midbrain and bridge, (d–f) hippocampus and (g–i) the prefrontal cortex by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Ovariectomy (OVX) increased tryptophan hydroxylase (TPH) expression levels in the midbrain and bridge (a) and 5-HT_{1A} receptor (5-HT_{1A}R) expression levels in the hippocampus (f). Administration of G-1 decreased serotonin transporter expression levels in the midbrain and bridge (a) (**$p < .01$, *$p < .05$, Games–Howell test). Box-and-whisker plots: boxes reflect the median, 25th quartile and 75th quartile. Whiskers reflect minimum and maximum values, and cross mark reflect mean.

et al., 2019). However, the function of GPR30 in modulating behaviour through the control of the serotonergic nervous system remains unclear. This study demonstrated that estradiol modulates the serotonergic nervous system via GPR30 during childhood in an OVX mouse model. We presented several lines of evidence that estradiol depletion in childhood alters social cognitive behaviour via GPR30 and that regulation of serotonin synthesis is involved in this behavioural change.

GPR30 regulates the endocrine, reproductive, immune, metabolic, cardiovascular and nervous systems (Prossnitz & Barton, 2011). We observed that the body weight of young OVX mice significantly increased from 6 weeks of age. In addition, the administration of G-1 had no effect on OVX-induced weight gain. OVX mice in adulthood have been reported to gain weight (Mann et al., 2020), and our data indicate that the effect of estradiol on weight gain is not influenced by age. A previous study indicated that GPR30 mRNA is expressed in fat and skeletal muscles (Mizukami, 2010), although reports on its effects on body weight have been inconsistent. Martensson et al. showed that female GPR30 knockout mice had slightly decreased body weight at 4 months of age (Mårtensson et al., 2009), whereas Davis et al. showed an increase in body weight at 13 weeks of age (Davis et al., 2014). Our data indicated that GPR30 does

not contribute to weight gain or loss until 10 weeks of age. These differences may be explained by the fact that weight adjustment induced by GPR30 is age-dependent. Similar to the weight results, the administration of G-1 did not suppress OVX-induced uterine weight loss. GPR30 has been reported to be involved, in part, in uterine proliferation, possibly through crosstalk with ERα (Gao et al., 2011; Otto et al., 2009). Our data suggest that GPR30 stimulation alone does not promote uterine growth.

The regulation of brain by estradiol has been evaluated using various behavioural analyses in animal models. Although associations between estradiol and depression- and anxiety-like behaviours have been reported (Ishibashi et al., 2016; Rauhut & Curran-Rauhut, 2018; Wang et al., 2016), most studies have demonstrated the effects of estradiol in the menopausal period. This study focused on the behavioural effects of estradiol from weaning to adolescence and found similarities and differences with those observed in adulthood.

We showed that OVX at 4 weeks of age induced a decrease in spontaneous locomotor activity during the active (dark) period at 10 weeks of age. We previously reported that OVX at 9 weeks of age significantly reduced locomotor activity during the active period 8 weeks later (Furukawa et al., 2021). The suppression of spontaneous locomotor activity by a decrease in estradiol levels was consistent in both adulthood and childhood. In addition, G-1 administration ameliorated the OVX-induced decrease in spontaneous locomotion. Recent studies have reported that female GPR30 knockout mice aged 3–8 months are less active in home cages at night than wild-type mice (Kastenberger & Schwarzer, 2014). This is consistent with our data and suggests that GPR30 is involved in the regulation of activity levels under normal rearing conditions.

It has been suggested that the effects of estradiol on anxiety behaviour depend on many factors, including age (in weeks), dosage and type of behavioural testing (Borrow & Handa, 2017). We showed that childhood OVX induced excessive exploratory behaviour in the open field test and obsessive-compulsive behavioural tendencies in the marble-burying behaviour test. One form of anxious behaviour is assessed by the amount of activity in the central area in the open field test, and estradiol has been reported to suppress this behaviour in adult animal models (Furukawa et al., 2021; Taxier et al., 2022; Walf & Frye, 2010). In contrast, our data showed that OVX increased activity in in the central area and rearing behaviour (head stretches) and investigative sniffing of the surroundings (head bobs). The increase in OVX-induced obsessive-compulsive behaviour has been reported in previous studies using adult models (Mitra et al., 2016; Paris et al., 2014). Although our results did not show significant differences, we showed a trend towards compulsive behaviour similar to that of adult models. In addition, administration of G-1 had no effect on behavioural changes in either the open field test or the marble-burying behaviour test in OVX mice. Thus, lower estradiol levels in childhood may be associated with stereotypical and perseverative behaviours through pathways other than GPR30.

The involvement of sex hormones in social behaviour has been well-studied (Choleris et al., 2018; Ervin et al., 2015; Yoest et al., 2023). We found that the preference for novel mice over novel objects was high in all groups and was not affected by OVX. In contrast, we demonstrated that the preference for novel mice over familiar mice was an OVX-specific behaviour, which was low in the Sham group. Administration of G-1 also suppressed the OVX-induced increase in preference for novel mice over familiar mice. Karlsson et al. reported sex differences in social recognition, with female mice exhibiting lower social investigatory behaviour than male mice (Karlsson et al., 2015). However, it has been reported that the interest of female mice in novel mice is formed after puberty and that OVX-induced suppression of social behaviour is restored by G-1 administration (Yoest et al., 2023). These discrepancies may be due to differences in the number and duration of contact with the acclimatized mice prior to testing. In other words, contact time with acclimated mice plays a major role in whether they show interest in novel mice, suggesting that GPR30 may be involved in the loss of interest in acclimated mice. Although there were differences in behavioural expression depending on the study conditions, our data support the involvement of estradiol in the regulation of social cognitive behaviour in childhood through adolescence via GPR30.

One mechanism underlying the regulation of behaviour by estradiol involves the serotonergic nervous system. We have previously reported that decreased estradiol suppresses serotonin release and decreases spontaneous locomotor activity in an adult OVX animal model (Furukawa et al., 2021; Izumo et al., 2012). Previous studies have also reported that estradiol increases TPH expression in the raphe nucleus via ERβ (Hiroi & Handa, 2013) and enhances serotonergic transmission in the limbic areas and behaviour (Wharton et al., 2012). However, in our childhood model, OVX increased TPH mRNA expression in the region containing the raphe nucleus and increased serotonin release in the amygdaloid nucleus. Furthermore, increases in SERT expression and serotonin release were suppressed by G-1 administration, with a suppressive trend in TPH expression. These differences may be because the distribution of ER expression changes with age, which affects the regulation of the serotonergic nervous system. In addition, our data

indicate that GPR30 may act in a repressive manner on the expression of TPH as opposed to ERβ. Sugiyama et al. reported that the expression of ERα and ERβ differs between postnatal and adolescent stages (Sugiyama et al., 2009). However, the mechanism by which GPR30 expression changes during growth remains unclear.

In the hippocampus, OVX increased 5-HT_{1A}R expression, but G-1 administration had no effect. Although estradiol has been reported to induce desensitization of 5-HT_{1A}R signalling and GPR30 is involved in this desensitization in the paraventricular nucleus (D'Souza et al., 2004; McAllister et al., 2012), the regulation of 5-HT_{1A}R by estradiol may be brain region-specific. In addition, the results of the open field test and marble-burying behaviour test were consistent with the variation in the expression level of 5-HT_{1A}R in this study, suggesting the involvement of 5-HT_{1A}R in stereotypic and obsessive-compulsive behaviours in childhood. However, the findings of this study have to be seen in light of the limitation of the lack of protein quantity analysis. Future studies are needed to confirm GPR30-mediated changes in serotonin receptors over time by both gene expression and protein quantification.

Estradiol has been shown to modulate mesolimbic dopamine systems in female animal (Yoest et al., 2014). It has been reported that female rats were found to have higher extracellular striatal dopamine concentrations in proestrus and estrus than after OVX (Xiao & Becker, 1994). However, we found no significant difference in dopamine release in the amygdaloid nucleus or the prefrontal cortex in young OVX. These differences may be due to the region of brain or the week of surgery performed.

Taken together, our findings indicate that GPR30-mediated regulation of serotonergic system is involved in changes in activity and social-cognitive behaviour due to decreased estradiol levels in childhood. This study will improve our understanding of the involvement of estradiol in the mechanisms of psychiatric symptoms, such as developmental and anxiety disorders, in girls and women. It will be important for future research to determine the relationship between the distribution of GPR30 and serotonergic projections during brain development.

5 | CONCLUSION

We demonstrated that childhood OVX induces decreased spontaneous locomotor activity and changes in social-cognitive behaviour, which are suppressed by G-1 administration. Moreover, we found that childhood OVX increases serotonin release in the amygdala and that administration of G-1 decreases this release by inhibiting SERT. Our results suggest that estradiol modulates activity levels and social behaviour in female children by regulating the serotonergic nervous system via GPR30.

AUTHOR CONTRIBUTIONS

Megumi Furukawa: Conceptualization; data curation; methodology; project administration; writing—original draft; writing—review and editing. **Nobuo Izumo:** Conceptualization; data curation; funding acquisition; project administration; resources; supervision; writing—original draft; writing—review and editing. **Ryoken Aoki:** Data curation. **Daichi Nagashima:** Methodology. **Yukiko Ishibashi:** Methodology. **Hideo Matsuzaki:** Supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

We have no conflict of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author (H.M.).

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