Tris buffer were added. The absorbance was read at 405 nm every 15 s for 10 min. The rate of the absorbance increase (Δ405 nm/s/ml) was calculated as the amount of enzyme hydrolysis activity.

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Serotonin 2C receptor antagonism ameliorates novelty-induced hypophagia in aged mice

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Summary This study was conducted to clarify the role of serotonin (5-hydroxytryptamine, 5-HT) 2C receptor (5-HT_{2C}R) signaling during novelty-induced hypophagia in aged mice. Male C57BL/6J mice [6-week-old (young) and 79–80-week-old (aged) mice] were exposed to a novel environment, and its effects on feeding behavior, stress hormones, and appetite-related factors were examined. Exposure of aged mice to a novel environment suppressed food intake and increased corticosterone secretion. These responses were marked compared with those in young mice. The expression in hypothalamic corticotropin-releasing factor (CRF), pituitary CRF1R and proopiomelanocortin mRNA in aged mice exposed to a novel environment was increased or tended to increase, compared to control mice. 5-HT_{2C}R antagonist, SB242084 or rikkunshito administration attenuated the decrease in food intake and increased stress hormone levels in aged mice exposed to the environmental change. The 5-HT_{2C}R mRNA expression in paraventricular nucleus was significantly enhanced, when aged mice was exposure to the novel environment. Thus, novelty-induced hypophagia in aged mice resulted, at least in part, from up-regulated hypothalamic 5-HT_{2C}R function. In conclusion, 5-HT_{2C}R signaling enhancement and the subsequent activation of the CRF neuron were involved in novelty-induced hypophagia in aged mice, and the 5-HT_{2C}R antagonists offer a promising therapeutic option for depression.

1. Introduction

The number of older people with anxiety and depression is increasing as our society ages, and the treatment for these disorders continues to attract attention (Diefenbach and Goethe, 2006). Appetite loss is a characteristic of older

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people and can result in increased morbidity and progressive physical disability (Rowe and Kahn, 1987; Seeman and Robbins, 1994). Various factors contribute to decreased food intake among the older people, and psychological factors resulting from responses to social and environmental changes are especially important (Donini et al., 2003; Hughes et al., 2004). Late-life events such as interpersonal loss and bereavement particularly appear to be associated with the onset and relapse of depression (Lenze et al., 2001). Thus, the combination of anxiety and aging are major factors regulating food intake.

The neural mechanisms by which environmental change suppresses feeding behavior remain unclear. Activation of the brain corticotropin-releasing factor receptors (CRFRs) may influence appetite, gastrointestinal function, and emotional responses (Hotta et al., 1999; Zorrilla et al., 2003). In addition to their major role in activating the hypothalamo–pituitary–adrenal (HPA) axis (Itoi et al., 2004), CRFRs are involved in stress responses including anxiety and decreased food intake (Hotta et al., 1999). Acute hypophagia upon exposure to a novel environment is due to CRF1R activation (Saegusa et al., 2011). Food intake and anxiety are also regulated by other neurotransmitters, including serotonin (5-hydroxytryptamine, 5-HT), which is involved in emotional and feeding behaviors and other physiological responses. Acute 5-HT depletion acts to reduce anxiety such as increased latencies to approach food and decreased food intake in novelty-induced hypophagia (Bechtholt et al., 2007).

Of the 5-HT receptor subtypes, 5-HT_{2C}R, which is localized on the proopiomelanocortin (POMC) neurons of the arcuate nucleus (ARC) and CRF neurons of the paraventricular nucleus (PVN), stimulates anxiety (Heisler et al., 2007b) and 5-HT_{2C}R activation increases anxiety-like behaviors (Gatch, 2003; de Mello Cruz et al., 2005). In addition, 5-HT_{2C}R negatively regulates food intake (Dryden et al., 1996; Heisler et al., 2002; Hayashi et al., 2005; Nonogaki, 2008). Recent studies have reported that 5-HT_{2C}R and CRF interact (Hemrick-Luecke and Evans, 2002; Saegusa et al., 2011); however, the participation of CRF and 5-HT_{2C}R during environmental changes of the aging animal remain unclear.

Decreased food intake in aging rodents has been confirmed by other groups and us (Toshinai et al., 2007; Takeda et al., 2010); however, no study has determined the influence of exposure to a novel environment on feeding behavior in aged animals. Evaluation of anxiogenic conditions in animal models is generally conducted by an open field test or examining feeding behaviors in novel environments such as an unfamiliar cage or an anxiogenic environment. Animals exhibiting novelty-induced inhibited feeding may be responsive to antidepressants, and the inhibition is determined by measuring feeding latency and/or the amount of food consumed in a novel environment (Dulawa and Hen, 2005; Bechtholt et al., 2007). In the present study, we evaluated clinically recognized stress caused by environmental changes and social isolation by not only exposure to novel environment but also removing the animals from group housing and placing them in isolation. Therefore, animals can be used to clinically evaluate depression and anxiety. We hypothesized that decreased food intake in aged mice exposed to a novel environment occurs via a mechanism involving the serotonergic nervous system, specifically continuous 5-HT_{2C}R activation. To test our hypothesis, we subjected aged mice to a novelty hypophagia paradigm and examined the effect of a selective 5-HT_{2C}R antagonist and Japanese kampo medicine, rikkunshito (RKT) on these variables to clarify the association between decreased feeding behavior and 5-HT_{2C}R in aged mice after exposure to a novel environment. In addition, to clarify the mechanism of increased function in 5-HT_{2C}R, we investigated the change in the 5-HT_{2C}R mRNA expression in the hypothalamus.

2. Methods

2.1. Animals

Male C57BL/6J mice, aged 6 weeks (young mice; 22.8 ± 0.3 g BW) and 79–80 weeks (aged mice; 44.1 ± 0.6 g BW), were purchased from Charles River Laboratories Japan, Inc., Tokyo, Japan. We used mice before aging cachexia. Before the experiment, mice were acclimated in 5 mice per cage condition in a temperature and humidity-controlled room under a 12-h (07:00 h–19:00 h) light cycle and free access to food and water. The novelty-induced hypophagia test was modified from the methods described by Merali et al. (2004) and Bechtholt et al. (2007). The mouse challenge test was performed by suddenly transferring group-housed mice (5 mice/cage; cage size W230 × D310 × H155 mm) to separate cages (1 mouse/cage; cage size W136 × D208 × H115 mm) to evaluate the suppression of feeding by exposure to a novel environment (i.e., isolation). Before beginning the experiments, control mice (home-cage mice) were housed in separate cages for 7 days. All experiments were performed between 09:00 h and 18:00 h. This study was approved by and conducted according to the guidelines of the experimental animal ethics committees of Tsumura & Co., Ibaraki, Japan (permit no.: 08-212).

2.2. Effect of exposure to novel environment on serum adrenocorticotropic hormone (ACTH) and corticosterone secretions and food intake

To determine ACTH and corticosterone levels, blood was collected via the carotid artery of each mouse during freely fed condition 24 h after exposure to the novel environment (n = 4–5). Samples were collected between 13:00 h and 15:00 h to avoid diurnal ACTH and corticosterone variations. Corticosterone levels were measured using the Corticosterone EIA Kit (Enzo Life Sciences., Plymouth Meeting, PA, USA), and ACTH levels were measured using the ACTH EIA Kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA).

The effect of exposure to a novel environment on food intake were evaluated 6 and 24 h after isolation (n = 8–10). Food intake was defined as the difference between the weights of a standard chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) before examination and that of the food recovered subsequent to the test session at each time point. Mice received ad libitum access to water during the experiments. Spilled food was collected, combined with the remaining food, and added to the total weight. Provisional food intake by group-housed mice was calculated to ensure that no differences were observed between group-housed and control mice, which were in separate

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cages for 7 days. Mean food intake by group-housed mice was determined by dividing food intake by the number of mice per cage (5 mice/cage).

2.3. Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis

The hypothalamus and pituitary gland were rapidly removed from each mouse in freely fed status 24 h after exposure to the novel environment (n = 5–13) and immediately frozen in a tube on dry ice. Isolated tissue homogenization and total RNA extraction were performed using the RNeasy Universal Tissue Kit (Qiagen, Valencia, CA, USA). Each sample was then diluted to 100 ng/μL. Diluted total RNA was incubated at 70 °C for 5 min and then cooled on ice. Total RNA (1000 ng) was reverse transcribed using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR assays were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) on a Prism 7900HT Sequence Detection System (Applied Biosystems). mRNA expression was normalized using ribosomal protein S29 as an endogenous control to correct the differences in the amount of total RNA added for each reaction. Differences were expressed by the dCt (Ct, threshold cycle) value, i.e., $dCt = 2^{(-|A-B|)}$, where A and B are the number of cycles needed by the housekeeping and target genes, respectively, to reach the threshold. All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were manufactured by Applied Biosystems (*Rps29*, Mm02342448_gH; *Crh*, Mm01293920_s1; *Crhr1*, Mm00432670_m1; *Htr2c*, Mm00434127_m1; *Npy*, Mm00445771_m1; *Agrp*, Mm00475829_g1; *Pomc*, Mm00435874_m1). Primers and probe sets that were used to detect the CRF primary transcript (CRF hnRNA) were designed using the Custom TaqMan Assay Design Tool (Applied Biosystems). The sequence of the forward primer was GGAATGGAGACAGAGAAGGTTGTTC and that of the reverse primer was AGCTGTCGCACACCCTAATC.

2.4. Effects of SB242084 and RKT on food intake

To clarify the role of 5-HT_{2C}R on decreased food intake after a novel environmental change, we investigated the effects of 5-HT_{2C}R antagonists on food intake in isolated young and aged mice. The selective 5-HT_{2C}R antagonists SB242084 (6 mg/kg PO; Tocris Bioscience, Glasgow, UK) (Hayashi et al., 2005) and RKT (1000 mg/kg PO; Tsumura & Co., Tokyo, Japan) (Takeda et al., 2008) were administered to mice under ad libitum feeding conditions immediately after isolation (n = 5–8). Control animals were administered PO with distilled water in place of test drugs. Oral administration of SB242084 (6 mg/kg) to young and aged control mice (non-novel environmental change) was performed to clarify the role of 5-HT_{2C}R on basal feeding behavior. To confirm the antagonistic actions of RKT on 5-HT_{2C}R activation, 1-(m-chlorophenyl) piperazine (mCPP; Sigma–Aldrich, St. Louis, MO, USA) was administered to young (5 mg/kg IP) and aged mice (3 mg/kg IP) (Lee et al., 2004), and RKT was simultaneously administered at a dosage of 1000 mg/kg PO. The food intake was determined as mentioned above. In addition, to clarify the changes in 5-HT_{2C}R function in aged mice, IP injection of mCPP at doses of 1, 3, or 5 mg/kg was administered to young and aged mice and the food intake was also determined 3 or 6 h after mCPP treatment.

2.5. Effects of SB242084 and RKT on serum stress hormone secretion and stress- and appetite-associated peptide mRNA expression

We also investigated the effect of SB242084 (6 mg/kg) and RKT (1000 mg/kg) on serum ACTH and corticosterone concentrations as well as hypothalamic or pituitary mRNA expression in control mice and those exposed to the novel environment. Each test drug was administered to the ad libitum fed mice, which were simultaneously isolated, and blood was collected 24 h after exposure to the novel environment. To investigate mRNA expression, the hypothalami and pituitary glands of 24-h fasted mice were collected 6 h after exposure to the novel environment.

2.6. In vitro assay for 5-HT_{2C}R

CHO-K1 cells stably transfected with a plasmid encoding the human 5-HT_{2C}R were used to prepare membranes in modified Tris-HCl buffer. A membrane protein was incubated with 1.0 nmol/mL [^{3}H]-mesulergine for 60 min at 25 °C. Non-specific binding was estimated in the presence of 1 μmol/L mianserin (Nippon Organon K.K., Osaka, Japan). The filters were then assayed for radioactivity to determine the amount of specifically bound [^{3}H]-mesulergine (Wolf and Schutz, 1997).

The antagonistic activity of compounds on human 5-HT_{2C}R expressed in transfected HEK-293 cells was determined by measuring their effects on agonist-induced IP_1 production using the HTRF detection method (Porter et al., 1999). Cells were suspended in buffer [10 mM Hepes (pH 7.4)], plated in 96-well microplates at a density of 2×10^4 cells/well, and preincubated for 5 min at room temperature in the presence of buffer (basal control) or the test compound. Thereafter, the reference agonist 5-HT was added at a final concentration of 10 nM. Separate assay wells did not contain 5-HT for basal control measurements. After 30 min incubation at 37 °C, cells were lysed and the fluorescence acceptor (D2-labeled IP_1) and donor (anti-IP_1 antibody labeled with europium cryptate) were added. After 60 min incubation at room temperature, the fluorescence transfer was measured at λ_{ex} = 337 nm and λ_{em} = 620 and 665 nm. IP_1 concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm. Results are expressed as the percent inhibition of the control response to 10 nM 5-HT. A concentration–response curve was generated to calculate IC_{50} values (Porter et al., 1999).

2.7. In situ hybridization (ISH)

For ISH, paraffin-embedded blocks and sections of 3 or 4 mice brains in each age group were obtained from Genostaff, Inc. (Tokyo, Japan). The brains of young and aged mice that were exposed to the novel environment for 6 h in a freely fed state were dissected, fixed with Tissue Fixative (Genostaff, Cat. No. TSF-01), embedded in paraffin by a proprietary procedure, and sectioned at a thickness of 6 μm. For the ISH study,

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Table 1 Effect of exposure to novel environment on serum adrenocorticotropic hormone (ACTH) and corticosterone levels.

	Young		Aged	
	Control	Isolation	Control	Isolation
ACTH (ng/mL)	0.42 ± 0.10	0.80 ± 0.04$^{\#}$	0.83 ± 0.06*	1.42 ± 0.11$^{***,\#\#\#}$
Corticosterone (ng/mL)	58.7 ± 12.7	97.6 ± 44.9	253.8 ± 87.8	2069.4 ± 743.4$^{**,\#\#}$

Data are presented as mean ± SEM (n = 4–5).
* $P < 0.05$ vs. young mice.
** $P < 0.01$ vs. young mice.
*** $P < 0.001$ vs. young mice.
$^{\#}$ $P < 0.05$ vs. age-matched control mice.
$^{\#\#}$ $P < 0.01$ vs. age-matched control mice.
$^{\#\#\#}$ $P < 0.001$ vs. age-matched control mice.

oligonucleotide RNA probes with a length corresponding to 654 base-pair fragments were designed from positions 117–830 of the mouse 5-HT_{2C}R cDNA (GenBank accession number NM_008312.4). Hybridization was performed under the contract of Genostaff. The RNA probes were labeled with digoxigenin (Roche Molecular Biochemicals, Mannheim, Germany) and hybridized at 60 °C for 16 h. The bound label was detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Sigma–Aldrich), and the tissue sections were stained with Kernechtrot staining solution (Muto Pure Chemicals, Tokyo, Japan).

2.8. Statistical analyses

Statistical analyses of mean values of 2 groups were performed using the Student's t-test. Differences in multiple group mean values were assessed by two-way factorial analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Data are presented as mean ± standard error for each group and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of exposure to novel environment on ACTH and corticosterone secretions and food intake

All observations pertaining to mice exposed to the novel environment were made 24 h after exposure. Serum ACTH levels in aged control mice were greater than those in young control mice. Exposure to the novel environment resulted in an approximately two-fold increase in serum ACTH levels in both young and aged mice (Table 1). Two-way factorial ANOVA revealed that the effects of treatment [$F(1, 16) = 40.02$] and age [$F(1, 16) = 36.03$] were significant; however, no significant effect of treatment × age was observed. At basal conditions, serum corticosterone levels in aged control mice tended to be higher than those in young control mice. Similar to the changes in serum ACTH levels, serum corticosterone levels in aged mice increased much more markedly than those in young mice after exposure to the novel environment (Table 1). Two-way factorial ANOVA revealed that the effects of treatment [$F(1, 13) = 11.93$], age [$F(1, 13) = 15.31$], and treatment × age [$F(1, 13) = 11.15$] were significant.

No significant difference in food intake was observed between the control groups (young, 5.3 ± 0.1 g/24 h; aged, 4.9 ± 0.3 g/24 h) 7 days after isolation and group-housed mice (young, 4.6 ± 0.2 g/24 h; aged, 4.5 ± 0.4 g/24 h). In young mice, food intake was only slightly decreased at 6 and 24 h after exposure to the novel environment (Table 2). In contrast, food intake in aged mice was markedly decreased after isolation. With regard to 6-h food intake, two-way factorial ANOVA revealed that the effects of treatment [$F(1, 33) = 90.58$], age [$F(1, 33) = 54.38$], and treatment × age [$F(1, 33) = 36.68$] were significant. With regard to 24-h food intake, two-way factorial ANOVA revealed that the effects of treatment [$F(1, 33) = 63.60$], age [$F(1, 33) = 45.20$], and treatment × age [$F(1, 33) = 25.34$] were significant.

Table 2 Effect of exposure to novel environment on cumulative food intake.

Period	Cumulative food intake (g)			
	Young		Aged	
	Control	Isolation	Control	Isolation
6 h	1.60 ± 0.09	1.49 ± 0.09	1.31 ± 0.08	0.19 ± 0.07$^{\#\#\#}$
24 h	5.31 ± 0.10	4.50 ± 0.20$^{\#\#}$	4.88 ± 0.28	1.53 ± 0.34$^{\#\#\#}$

Data are presented as mean ± SEM (n = 8–10).
$^{\#\#}$ $P < 0.01$ vs. age-matched control mice.
$^{\#\#\#}$ $P < 0.001$ vs. age-matched control mice.

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Table 3 Effect of exposure to novel environment on pituitary and hypothalamic mRNA expression.

	Young		Aged	
	Control	Isolation	Control	Isolation
Pituitary mRNA relative expression				
CRF1R	1.00 ± 0.05	0.97 ± 0.12	0.88 ± 0.01	1.18 ± 0.08$^{\#}$
POMC	1.00 ± 0.24	1.29 ± 0.11	1.05 ± 0.06	1.84 ± 0.29
Hypothalamic mRNA relative expression				
CRF	1.00 ± 0.13	1.12 ± 0.08	0.63 ± 0.05	0.83 ± 0.04$^{\#\#}$
POMC	1.00 ± 0.09	1.25 ± 0.20	0.98 ± 0.10	1.27 ± 0.11
NPY	1.00 ± 0.09	0.85 ± 0.05	0.64 ± 0.05**	0.61 ± 0.01**
AGRP	1.00 ± 0.10	0.90 ± 0.11	0.54 ± 0.02**	0.53 ± 0.07*

Data are presented as mean ± SEM (n = 5–13).
* $P < 0.05$ vs. young mice.
** $P < 0.01$ vs. young mice.
$^{\#}$ $P < 0.05$ vs. age-matched control mice.
$^{\#\#}$ $P < 0.01$ vs. age-matched control mice.

3.2. Effect of exposure to novel environment on stress- and appetite-associated peptide mRNA expression

To clarify the association between anxiety and feeding in mice exposed to the novel environment, we investigated the effect of 24-h exposure to the novel environment on the hypothalamic CRF system. In young mice, hypothalamic CRF mRNA and pituitary POMC mRNA did not change 24 h after exposure to the novel environment. In contrast, in aged mice, hypothalamic CRF mRNA and pituitary CRF1R mRNA significantly increased and pituitary POMC mRNA tended to increase after isolation (Table 3).

Next, we examined hypothalamic gene expression of appetite-regulating neuropeptides in young and aged mice with and without exposure to the novel environment. In the control condition, hypothalamic NPY and AGRP mRNA expression levels in aged mice were significantly lower compared with those in young mice, while POMC mRNA expression levels were comparable. Reduction in NPY and AGRP mRNA levels in aged mice was observed even after isolation (Table 3).

3.3. Effect of SB242084 on food intake, serum stress hormone secretion, and stress- and appetite-associated peptide mRNA expression in aged mice exposed to novel environment

To clarify the role of 5-HT_{2C}R on decreased food intake after the novel environmental change, we investigated the effect of SB242084, a 5-HT_{2C}R antagonist, on food intake. As shown in Fig. 1A, oral administration of SB242084 at a dosage of 6 mg/kg to young mice did not affect basal food intake 3–24 h after the treatment. Similarly, differences in food intake between aged mice receiving SB242084 and those receiving only distilled water were not significantly different until 6 h after the treatment, although SB242084 slightly increased cumulative food intake after 24 h (Fig. 1B). In contrast, after exposure to the novel environment, the administration of SB242084 significantly attenuated novelty-induced hypophagia in aged mice over the experimental period (Fig. 1B).

5-HT_{2C}Rs are reportedly expressed in both CRF neurons in PVN (Heisler et al., 2007a) and POMC neurons in ARC (Xu et al., 2008). To elucidate the role of these 5-HT_{2C}Rs in the hypothalamus in aged mice, the effects of SB242084 on the HPA axis and the expression of hypothalamic appetite-regulating peptides were investigated. After exposure to the novel environment, serum ACTH and corticosterone levels were significantly increased, and the administration of SB242084 attenuated these responses (Fig. 2A and B). Hypothalamic CRF mRNA expression was significantly increased in SB242084-treated mice compared with that in distilled water-treated and isolated mice (distilled water-treated, 1.00 ± 0.06; SB242084-treated, 1.41 ± 0.09; $P < 0.01$; data not shown in figures and tables). A similar change was observed in hypothalamic CRF hnRNA expression (distilled water-treated, 1.00 ± 0.07; SB242084-treated, 1.42 ± 0.10; $P < 0.01$; data not shown in figures and tables). Neither pituitary CRF1R nor POMC mRNA expression was altered by SB242084 treatment (CRF1R: distilled water-treated, 1.00 ± 0.06; SB242084-treated, 1.00 ± 0.12; $P = 0.98$; POMC: distilled water-treated, 1.00 ± 0.12; SB242084-treated, 1.10 ± 0.11; $P = 0.55$; data not shown in figures and tables).

In aged control mice, the administration of SB242084 (6 mg/kg PO) failed to affect basal serum corticosterone levels but suppressed basal hypothalamic CRF gene expression (Supplemental Figures S2, S3).

Hypothalamic NPY and AGRP but not POMC mRNA expression levels were significantly increased after the administration of SB242084 in aged mice (Fig. 2C–E).

3.4. Effect of RKT on food intake, serum stress hormone secretion, and stress- and appetite-associated peptide mRNA expression in aged mice exposed to novel environment

It has been demonstrated that RKT has a 5-HT_{2C}R antagonistic-like action both in vivo and in vitro (Takeda et al., 2008; Fujitsuka et al., 2009; Yakabi et al., 2010a,b). Therefore, we investigated the possibility that similar to SB242084, RKT would inhibit reduction in food intake in aged mice after

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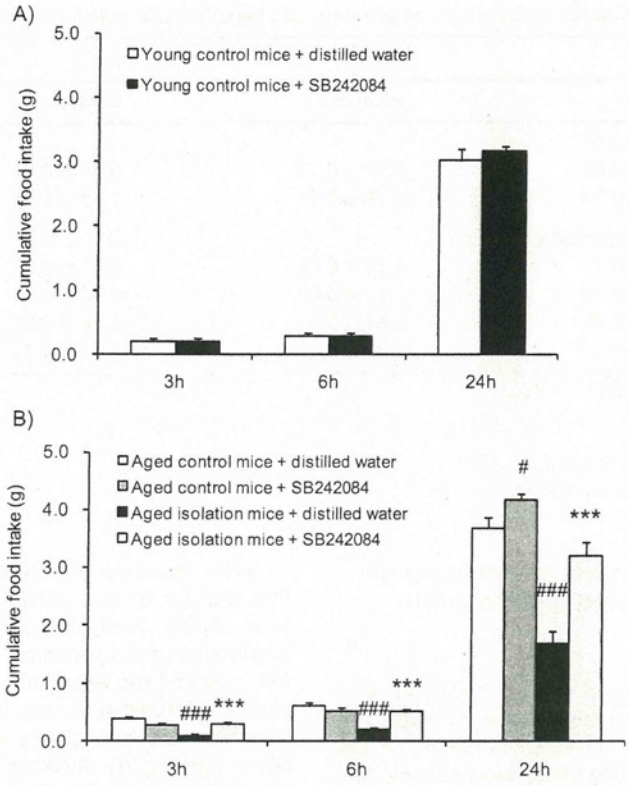

Figure 1 Effect of serotonin 2C receptor antagonist on food intake in young and aged mice; SB242084 (6 mg/kg PO) was administered to (A) young and (B) aged mice, and cumulative food intake was measured 3, 6, and 24 h after the drug treatment or isolation. Data are presented as mean ± SEM ($n = 5–8$). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. distilled water-treated aged control group; $^{***}P < 0.001$ vs. distilled water-treated aged isolated group.

environmental changes and found that this was indeed the case (Fig. 3A). Following this, to confirm whether RKT would Exhibit 5-H T_{2C}R antagonism in vivo, RKT was administered PO to mCPP-treated young (Fig. 3B) and aged mice (Fig. 3C). The results show that RKT completely inhibited decreased food intake in aged mice 3 and 6 h after mCPP injection. Next, we reconfirmed the in vitro action of various components included in RKT on 5-HT_{2C}R binding and signal transduction. Table 4 shows the 5-HT_{2C}R-binding inhibitory and cellular 5-HT_{2C}R activities of the crude drug components contained in RKT. Among 36 types of RKT components assayed, glycycoumarin, isoliquiritigenin, and 8-shogaol showed IC_{50} values of 17.1, 7.5, and 16.5 μmol/L, respectively, on binding between [^{3}H] mesulergine and 5-HT_{2C}R and 7.7, 5.5, and 36.9 μmol/L, respectively, on IP_1 production when the cells were stimulated by 5-HT. Although not shown in Table 4, SB242084 as a positive control showed an IC_{50} value of 0.8 and 3.9 nmol/L, respectively, in the binding and IP_1 production assays.

Oral administration of RKT significantly attenuated the novelty-induced increases in serum ACTH and corticosterone levels in aged mice exposed to the novel environment (Fig. 4A and B). However, RKT treatment did not affect serum corticosterone levels in aged control mice (Supplemental Materials 3). Similar to SB242084, RKT treatment tended to increase hypothalamic CRF mRNA expression in aged mice 6 h after exposure to the novel environment (distilled water-treated, 1.00 ± 0.06; RKT-treated, 1.24 ± 0.16; $P = 0.19$). Pituitary expression of CRF1R and POMC was not affected (CRF1R: distilled water-treated, 1.00 ± 0.06; RKT-treated, 0.89 ± 0.11; $P = 0.38$; POMC: distilled water-treated, 1.00 ± 0.12; RKT-treated, 0.96 ± 0.13; $P = 0.80$). Pituitary CRF1R, POMC, and hypothalamic CRF mRNA expression in aged control mice was not altered by RKT treatment (Supplemental Materials 2). Moreover, a concomitant increase in NPY and AGRP mRNA expression was observed in RKT-administered aged mice exposed to the novel environment (Fig. 4C and D). Hypothalamic POMC mRNA in aged mice after the novel environmental change was not altered by the administration of RKT (Fig. 4E).

3.5. Effects of mCPP on food intake in young and aged mice

We hypothesized that the beneficial effect of 5-HT_{2C}R antagonism on food intake and stress hormones in aged mice exposed to the novel environmental change may be mediated by the increased functioning of 5-HT_{2C}R. Therefore, we compared the influence of 5-HT_{2C}R activation on food intake in young and aged mice using mCPP as a 5-HT_{2C}R agonist. As depicted in Fig. 5A and B, mCPP at a dosage of 5 mg/kg (IP) significantly inhibited both 3- and 6-h cumulative food intake in both young and aged mice, whereas much lower doses of

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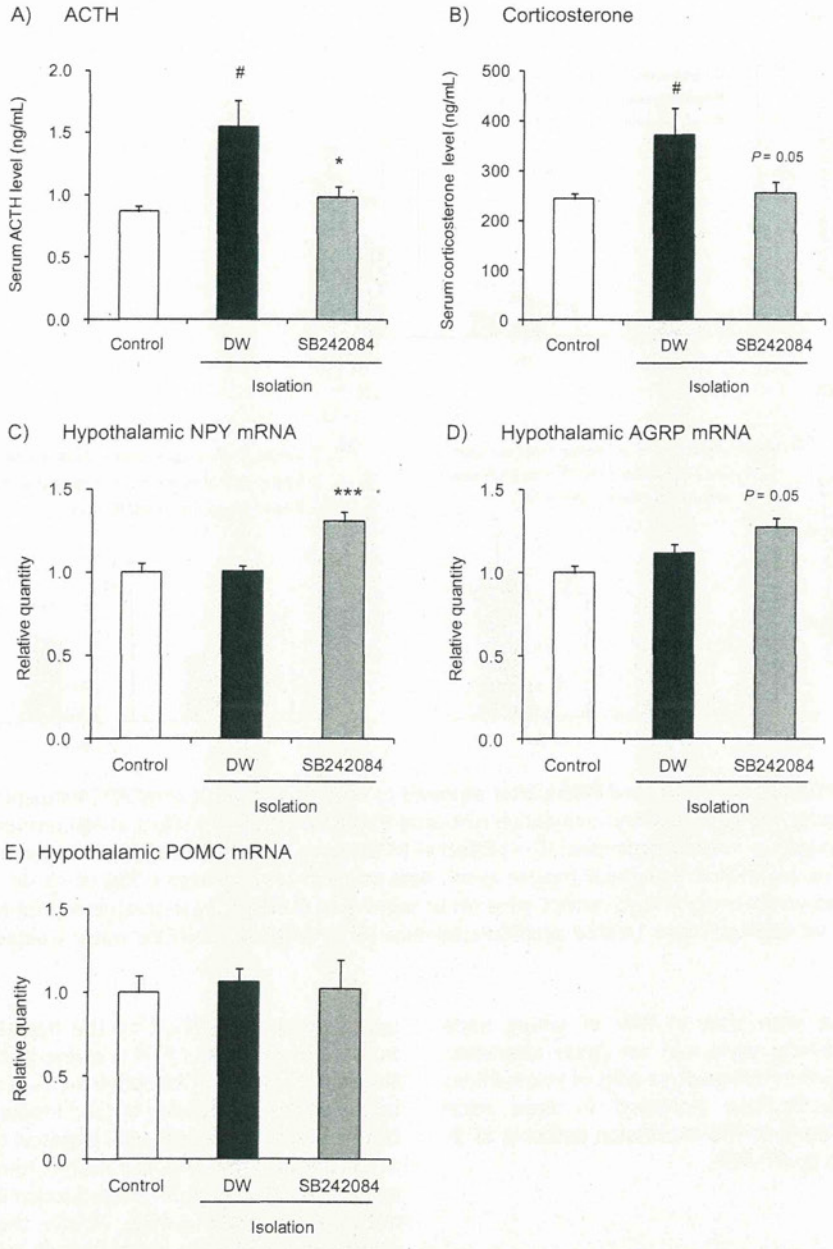

Figure 2 Effect of serotonin 2C receptor antagonist on stress hormone secretion and mRNA expression in aged mice exposed to novel environment; SB242084 (6 mg/kg PO) was administered to aged mice immediately after exposure to the novel environment. Blood and hypothalamus were collected 24 or 6 h after the isolation, respectively. (A) Serum adrenocorticotropic hormone (ACTH) and (B) corticosterone levels. (C) Hypothalamic mRNA expression of NPY, (D) AGRP, and (E) POMC. Data are presented as mean ± SEM (n = 5–8). DW: distilled water; $^{\#}P < 0.05$ vs. control group; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. DW-treated isolated group.

mCPP (1 or 3 mg/kg) failed to suppress food intake only in young mice.

3.6. Changes in 5-HT_{2C}R mRNA expression after exposure to novel environment

Because we observed a higher response to 5-HT_{2C}R antagonist and agonist in aged mice than that in young mice (Figs. 1 and 5), we hypothesized that the expression of 5-HT_{2C}R mRNA in PVN would be upregulated after isolation stress in aged mice. RT-PCR analysis revealed that 5-HT_{2C}R mRNA expression was not altered after exposure to the novel environment in young mice. In aged mice, however, hypothalamic 5-HT_{2C}R mRNA expression was significantly increased after novelty stress (Fig. 6A). To further confirm the upregulation of 5-HT_{2C}R mRNA expression in the hypothalamus of stress-loaded aged mice, we performed ISH in the hypothalamic PVN of both young and aged mice. No 5-HT_{2C}R mRNA was detected with the sense probe (data not shown in figures). In the absence of novelty stress, mRNA expression of 5-HT_{2C}R in PVN of aged

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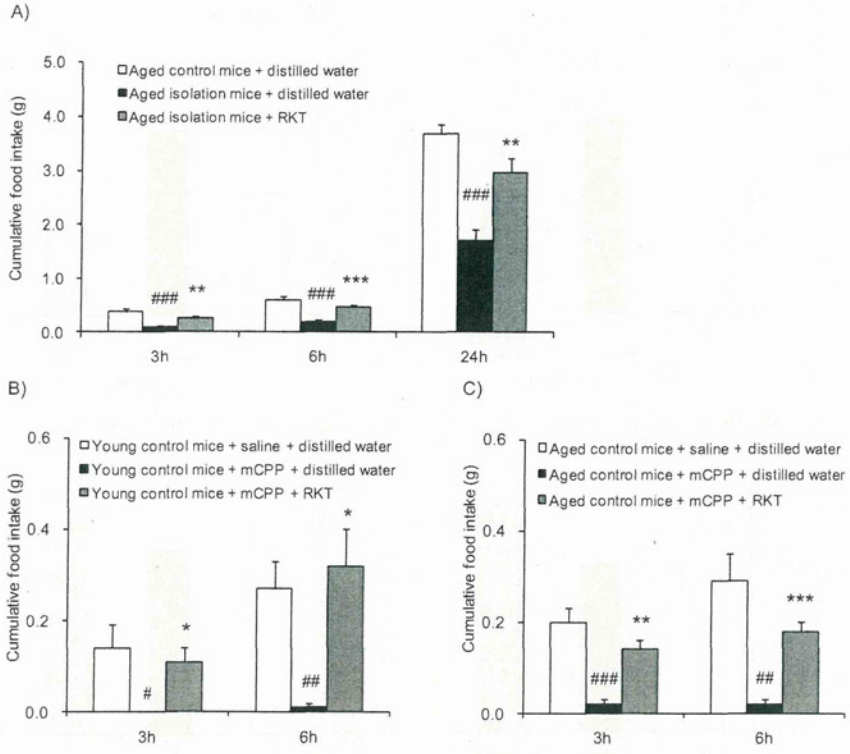

Figure 3 Effect of rikkunshito (RKT) on food intake after exposure to novel environment or mCPP treatment; RKT (1000 mg/kg PO) was administered to young and aged mice and cumulative food intake was measured. (A) Effect of RKT on cumulative food intake in aged mice after exposure to the novel environment. (B, C) Effect of RKT on cumulative food intake in young and aged mice after mCPP treatment (5 mg/kg IP to young mice, 3 mg/kg IP to aged mice). Data are presented as mean ± SEM ($n = 5$–8). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. distilled water-treated aged control mice (A) or saline and distilled water-treated control mice (B, C); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. distilled water-treated aged isolated mice (A) or mCPP and distilled water-treated control mice (B, C).

mice was comparable with that in PVN of young mice (Fig. 6B). Although novelty stress did not cause alteration in the number of 5-HT_{2C}R mRNA-positive cells in young mice, this number was significantly increased in aged mice (Fig. 6C). These data confirm the expression patterns of 5-HT_{2C}R mRNA detected by RT-PCR.

4. Discussion

In the present study, we found that exposure to a novel environment caused long-term stress hormone secretion and marked suppression of food intake in aged mice. This phenomenon can be mainly explained by the functional upregulation of 5-HT_{2C}R by the hypothalamus, specifically in the CRF neurons of PVN in stress-loaded aged mice. First, the administration of selective 5-HT_{2C}R antagonists markedly inhibited the suppression of food intake and hyperactivation of the HPA axis in aged mice exposed to the novel environment. Second, the administration of lower doses of a 5-HT_{2C}R agonist induced a significant reduction in food intake in aged mice but not in young mice. Finally, the isolation procedure caused an increase in hypothalamic 5-HT_{2C}R mRNA expression level in aged mice but not in young mice. From the above findings, we conclude that hypothalamic 5-HT_{2C}R plays a fundamental role in the regulation of food intake in aged mice under stress.

It is widely known that 5-HT_{2C}R activation induces anxiety-like behavior and appetite suppression in young mice (Dryden et al., 1996; Gatch, 2003; Hayashi et al., 2005; Halford et al., 2007; Nonogaki, 2008). However, no study has been conducted to elucidate the role of 5-HT_{2C}R under anxiogenic conditions in aged animals. In the present study, the administration of a selective 5-HT_{2C}R antagonist (SB242084), at a dose of 6 mg/kg that had no effect on food intake and stress hormone levels in young mice (Fig. 1A, Supplemental Figures S3, S4), significantly suppressed both long-term decrease in food intake and increase in stress hormone levels in aged mice exposed to the novel environment. The SB242084 dosage level of 6 mg/kg used in this

Table 4 Inhibitory effects of rikkunshito components for serotonin 2C receptor activity.

	IC_{50} (μmol/L)	
	Binding assay	Cell functional assay
Glycycoumarin	17.1	7.7
Isoliquiritigenin	7.5	5.5
8-Shogaol	16.5	36.9
Hesperetin	36.8	48.2

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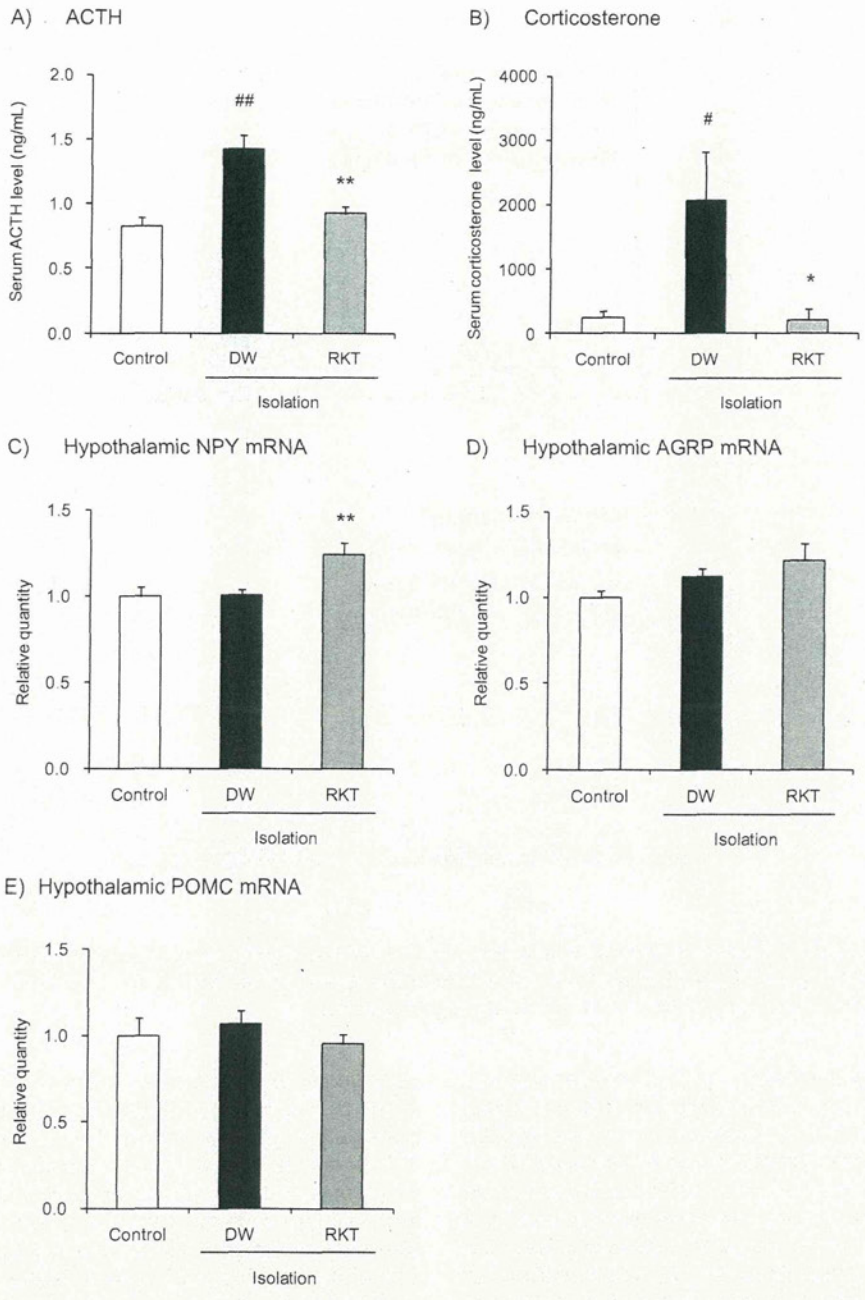

Figure 4 Effect of rikkunshito (RKT) on stress hormone secretion and mRNA expression in aged mice exposed to novel environment; RKT (1000 mg/kg PO) was administered to aged mice immediately after exposure to the novel environment. Blood and hypothalamus were collected 24 or 6 h after isolation, respectively. (A) Serum adrenocorticotropic hormone (ACTH) and (B) corticosterone levels. (C) Hypothalamic mRNA expression of NPY, (D) AGRP, and (E) POMC. Data are presented as mean ± SEM (n = 5–8). DW: distilled water; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. DW-treated isolated group.

study was similar to that required to antagonize the hypophagic response induced by mCPP in previous reports (Kennett et al., 1997), and it is not very different from the dosage of 0.1–3.0 mg/kg (IP) administered to evaluate anxiety (Martin et al., 2002). Because there was no effect of the SB242084 dosage level of 6 mg/kg on food intake in young control mice, we conclude that the administration of the 5-HT_{2C}R antagonist did not directly increase food intake in aged mice but rather inhibited the decrease in food intake by lowering the anxiety or stress response accompanying the novel environmental change.

Previous reports have noted that RKT exerts an antagonistic effect against 5-HT_{2C}R in vivo (Takeda et al., 2008; Fujitsuka et al., 2009; Yakabi et al., 2010a,b). Moreover, we have shown that RKT functions as an in vitro 5-HT_{2C}R inhibitor, possibly because of its constituents, which include

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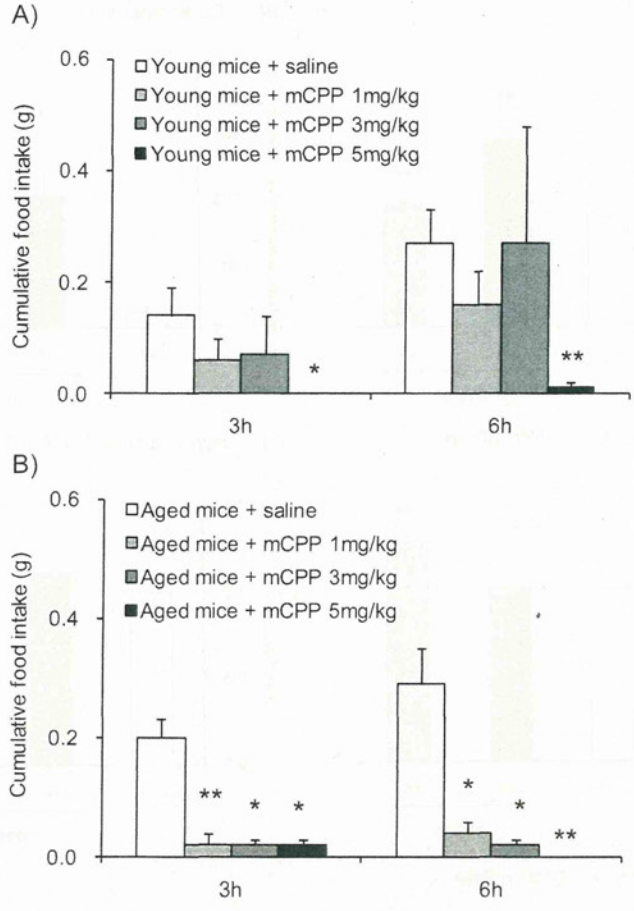

Figure 5 Effect of serotonin 2C receptor agonist on food intake in young and aged mice; Administration of mCPP (1, 3, or 5 mg/kg IP) was performed to (A) young and (B) aged mice. Cumulative food intake was measured at 3 or 6 h after mCPP injection. Data are presented as mean ± SEM ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. saline-treated mice.

glycycoumarin, isoliquiritigenin, and 8-shogaol (Takeda et al., 2008). Therefore, in this study, we tested whether RKT has the same effect as SB242084 on novelty-induced hypophagia in aged mice. We found that RKT attenuated not only HPA axis activation but also continuous decrease in food intake induced by exposure to the novel environment; this paralleled with the results obtained with SB242084. The present results also indicate that the administration of RKT inhibited mCPP-induced hypophagia in both young and aged mice, which is in agreement with previous findings (Yakabi et al., 2010a). Moreover, our in vitro cell functional assay indicated that certain constituents of RKT, including glycycoumarin, isoliquiritigenin, and 8-shogaol, exert inhibitory effects on cell signal transduction pathways involving 5-HT_{2C}R. IC_{50} values of the abovementioned components were 7.7, 5.5, and 36.9 μmol/L, respectively, and these values are very close to Ki values obtained by the receptor-binding assay (Takeda et al., 2008). These findings support the idea that RKT exerts an antagonizing effect on 5-HT_{2C}R in stress-loaded aged mice.

Next, we compared the influences of 5-HT_{2C}R activation on food intake in young and aged mice. Administration of the 5-HT_{2C}R agonist mCPP at a dosage of 5 mg/kg significantly inhibited both 3- and 6-h cumulative food intake in both young and aged mice, whereas much lower doses of mCPP (1 or 3 mg/kg) failed to suppress food intake only in young mice (Fig. 5A and B). These findings suggest that enhancement of the signal transduction pathway of 5-HT_{2C}R may occur in the brain of aged mice. This is analogous with our previous finding that decreased food intake in stress-loaded mice could be induced by lower dosages of the 5-HT_{2C}R agonist mCPP than those in non-stressed mice (Saegusa et al., 2011).

Although 5-HT_{2C}R is widely distributed in the brain, it is highly likely that the site of action of these two 5-HT_{2C}R antagonists is the CRF neuron-rich PVN because SB242084 markedly suppressed stress hormone release and decreased food intake after isolation. We found that an ICV injection of mCPP (50 μg/mouse) elevated serum corticosterone levels in young mice, as shown in Supplemental Materials (Figure S1). This is in agreement with a previous report that serum corticosterone levels were increased by mCPP administration and significantly suppressed by SB242084 (Hemrick-Luecke and Evans, 2002). Furthermore, a recent study has revealed that 5-HT_{2C}R is expressed in CRF neurons in PVN and that the 5-HT_{2C}R agonist activates these neurons, leading to ACTH release (Heisler et al., 2007a). The above findings support our hypothesis that stimulation of 5-HT_{2C}R in CRF neurons in PVN

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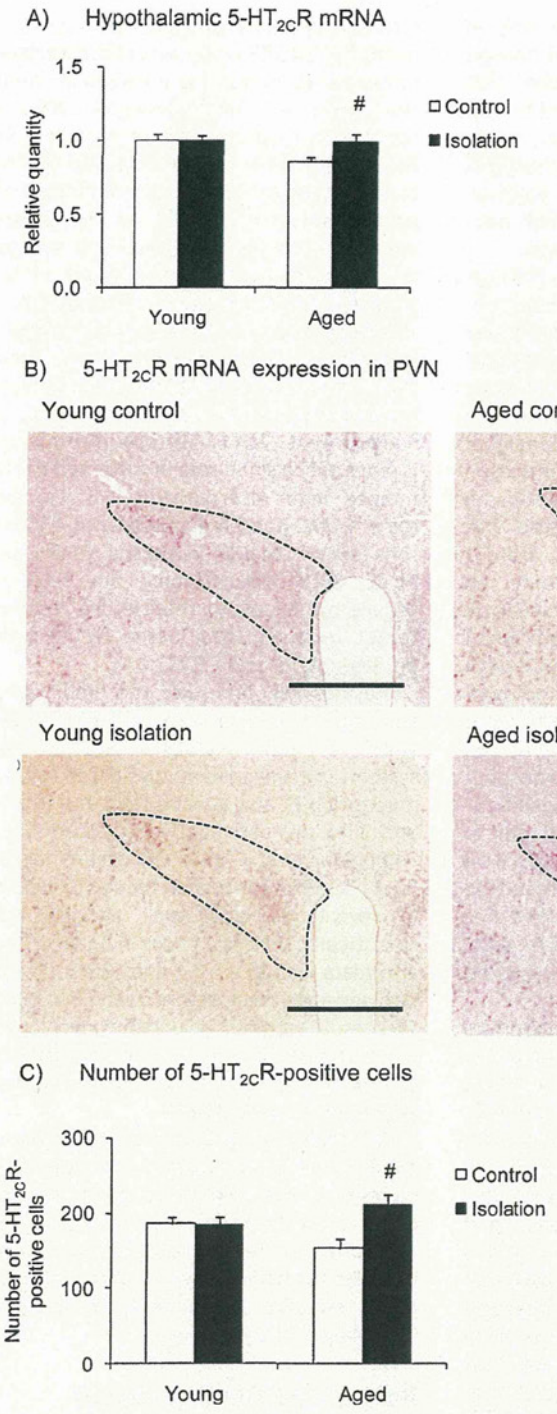

Figure 6 Hypothalamic relative expression and histological distribution of serotonin 2C receptor (5-HT_{2C}R) mRNA; Young and aged mice were exposed to the novel environment for 6 or 24 h in a freely fed condition. (A) Relative expression of hypothalamic 5-HT_{2C}R mRNA detected by reverse transcription polymerase chain reaction 24 h after isolation. (B) 5-HT_{2C}R mRNA expression in the paraventricular nucleus (PVN) detected by in situ hybridization 6 h after isolation. (C) Comparison of the number of 5-HT_{2C}R-positive cells in the PVN. Data are presented as mean ± SEM (n = 3–5). Bar: 500 μm. $^{\#}P < 0.05$ vs. aged control mice.

leads to the activation of the HPA axis observed in aged mice after novelty stress.

Our quantitative RT-PCR analysis revealed a significant increase in 5-HT_{2C}R gene expression in the hypothalamus of aged mice 24 h after exposure to a novel environment. To further confirm this observation, we performed ISH for 5-HT_{2C}R mRNA in young and aged mice with or without stress. We found that novel environmental stress caused a signifi-

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cant increase in 5-HT_{2C}R mRNA expression in PVN only in aged mice, suggesting that a novel environmental change may enhance 5-HT_{2C}R signaling in PVN in aged mice. This observation is consistent with our conclusion based on the results of a pharmacological approach. In contrast, novel environmental stress did not cause any significant change in 5-HT_{2C}R mRNA expression in other hypothalamic regions, including ARC, where appetite-regulating NPY/AGRP neurons and POMC neurons are located (data not shown).

It is plausible to consider that an increase in 5-HT_{2C}R mRNA in PVN may be the primary mechanism underlying 5-HT_{2C}R hyperfunction in aged mice. Nevertheless, there may be other possibilities: an increase in synaptic 5-HT concentration, an increase in receptor numbers and/or their affinity for 5-HT, decreased receptor desensitization and/or downregulation, and changes in intracellular signal transduction systems. In aged rodents, 5-HT concentration is reportedly increased by the inhibition of 5-HT turnover in the brain due to the environmental change compared with young rodents (Miura et al., 2002). Although we did not measure 5-HT concentration in the present study, an increase in 5-HT concentration at the 5-HT_{2C}R synaptic cleft in the brain of aged mice after exposure to a novel environment may play a role in 5-HT_{2C}R activation. As with other G protein-coupled receptors (GPCRs), functioning of 5-HT_{2C}R is fine-tuned by desensitization or internalization, which can be induced by GPCR kinases and arrestins (Van Oekelen et al., 2003). Therefore, 5-HT_{2C}R signal transduction may be enhanced by changes in receptor desensitization and/or internalization in stressed aged mice. Moreover, 5-HT_{2C}R is currently the only known GPCR in which pre-mRNA can be subject to RNA editing, resulting in alteration in its function (Burns et al., 1997). Whether these mechanisms are involved in the functional upregulation of 5-HT_{2C}R in stress-loaded aged mice remains to be determined.

In our study, the administration of 5-HT_{2C}R antagonists significantly inhibited stress hormone secretion in aged and stressed mice. Therefore, it was expected that the blockade of 5-HT_{2C}R would inhibit hypothalamic CRF, pituitary POMC, and CRF1R gene expression, all of which increased continuously up to 24 h after novel environmental changes were introduced in aged mice. However, contrary to our expectations, the administration of SB242084 or RKT in aged and stressed mice increased hypothalamic CRF mRNA, albeit it significantly decreased plasma ACTH and corticosterone levels. Although the exact reason for these unexpected increases in hypothalamic CRF mRNA after the administration of SB242084 and RKT is currently unknown, this suggests that stress hormone reduction and improved food intake due to 5-HT_{2C}R antagonism cannot simply be explained by the inhibition of hypothalamic CRF and pituitary POMC synthesis. Considering that there is a time lag between stress hormone secretion and hypothalamic CRF or pituitary POMC gene expression, the timing of sample collection should be verified in more detail.

Apart from CRF neurons in PVN, 5-HT_{2C}R is also found in POMC neurons in ARC, and it is believed to induce depolarization and increased POMC gene expression (Heisler et al., 2002), leading to the release of α-melanocyte-stimulating hormone (α-MSH). α-MSH binds to MC4R in CRF neurons in PVN and orexin neurons in lateral hypothalamic area, strongly suppressing appetite (Elmquist, 2001). On the other hand, NPY/AGRP is the best characterized and probably the most important peptide involved in stimulating food intake (Halford et al., 2007; Nonogaki, 2008) via either NPY Y1 receptor activation or antagonism of α-MSH to MC4R. Thus, feeding behavior is regulated by the balance of activity among these excitatory and inhibitory appetite regulation pathways (Tecott, 2007). In the present study, neither novelty stress per se nor 5-HT_{2C}R antagonism by SB242084 or RKT caused significant changes in hypothalamic POMC gene expression level. In contrast, the administration of SB242084 and RKT enhanced NPY/AgRP mRNA expression in aged mice after isolation stress. These findings suggest that in both young and aged mice, 5-HT_{2C}R in POMC neurons in ARC peaks at the acute phase (e.g., approximately 3 h; Saegusa et al., 2011) after the introduction of stress and that it plays only a small role in sustained decrease in food intake in aged mice. Alternatively, it is also possible that in aged mice, POMC neuron dominance in ARC stimulates CRF neurons in PVN through increased α-MSH secretion (Tachibana et al., 2007). Pharmacologically, 5-HT_{2C}R antagonism may reduce the excessive release of stress hormones and counteract reduced food intake by normalizing the balance between AgRP and POMC.

Besides PVN and ARC, the limbic system (including the amygdala) is another important brain region related to stress and food intake. Among the various regions of the limbic system, the amygdala is considered to be a key region to the perception of anxiety and fear, and it is also where 5-HT_{2C}R and CRFR are abundantly expressed (Swanson et al., 1983; Pompeiano et al., 1994). Our preliminary studies showed that 5-HT_{2C}R mRNA expression tended to increase in the amygdala in stress-loaded aged mice, although the results were not statistically significant (the number of positive cells in the amygdala was 85 ± 16.1 and 135 ± 18.4 in control aged and stressed aged mice, respectively; $P = 0.087$, data not shown). This result suggests the possible involvement of the amygdala in novelty-induced hypophagia. Further studies are required to clarify the role of the limbic system in stress-related alteration in food intake.

In conclusion, exposure of aged mice to a novel environment leads to a sustained decrease in food intake and increase in stress hormone levels via 5-HT_{2C}R activation. 5-HT_{2C}R antagonists such as SB242084 and RKT showed an ameliorative effect on reduction in food intake and secretion of stress hormones. These findings indicate that excessive 5-HT_{2C}R stimulation is deeply involved in novelty-induced hypophagia in aged mice.

Role of the funding source

There was no impact from funding source(s) on any aspect of the work with the current manuscript (design, data collection, analysis, interpretation, writing, or submission).

Conflict of interest

Dr. Takeda received grant support from Tsumura & Co. Dr. Iizuka, Dr. Sadakane, Ms. Saegusa, Ms. Nahata, and Dr. Hattori are employed by Tsumura & Co. Dr. Muto., Dr. Nakagawa, Dr. Ohnishi, and Dr. Asaka have no conflict to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.03.014.

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Review

Pathophysiological function of oxytocin secreted by neuropeptides: A mini review

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Abstract

Oxytocin (OXT) is well known for its ability to stimulate milk ejection and uterine contraction. OXT is also involved in several physiological and pathological functions such as antinociception, anxiety, feeding, social recognition and stress responses. Previous studies showed that neuropeptides such as cholecystokinin (CCK) activate OXT-secreting magnocellular neuron in the supraoptic (SON) and the paraventricular nuclei (PVN) of the hypothalamus and cause OXT release from the axon terminal in the posterior pituitary into the systemic circulation. Our recent studies showed that central administration of adrenomedullin (AM) family (AM, AM2 (identical to intermedin) and AM5) induced the expression of the c-*fos* gene in the SON and the PVN and elicited the marked increase of plasma OXT levels in conscious rats. Here, we review pathophysiological properties of OXT in whole body and effects of novel peptides such as AM family as well as other peptides on OXT release.

Keywords: Adrenomedullin family; Cholecystokinin; Hypothalamus; Neuropeptides; Paraventricular nucleus; Supraoptic nucleus

Contents

Abbreviations: AM, adrenomedullin; α-MSH, α-melanocyte stimulating hormone; AP, area postrema; AVP, arginine vasopressin; AVPV, the anteroventral periventricular nucleus; BNST, the bed nucleus of the stria terminalis; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; CeA, the central nucleus of the amygdala; CGRP, calcitonin gene-related peptide; CNS, central nervous system; LHA, the lateral hypothalamic area; NAcc, the nucleus accumbens; NTS, the nucleus of the solitary tract; OLETF, Otsuka Long-Evans Tokushima Fatty rat; OTR, oxytocin receptor; OXT, oxytocin; POMC, proopiomelanocortin; PrRP, prolactin-releasing peptide; PVN, the paraventricular nucleus; RFRP, RFamide-related peptides; SON, the supraoptic nucleus; VLM, the ventrolateral medulla; VMH, the ventromedial nucleus of hypothalamus.

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1. Introduction

Oxytocin (OXT), a nine amino acid neuropeptide, was discovered in 1906 as the extracts with uterus-contracting effects from pituitary [1]. OXT was the first peptide hormone to be sequenced and synthesized in 1953 [2–4]. OXT and Arginine vasopression (AVP) are closely related peptides synthesized primarily in magnocellular neurons of the hypothalamus localized in the supraoptic (SON) and the paraventricular nuclei (PVN), which project their axon terminals into the posterior pituitary where it is released into the systemic circulation [5,6].

OXT is well known for its roles in reproduction, especially during and after childbirth. A large amount of OXT is released after distension of the cervix and uterus during labor to help birth, and after stimulation of the nipples to milk ejection. Previous many studies showed that OXT is involved in several of physiological and pathological functions such as antinociception, anxiety, feeding, social recognition and stress responses [7–12] (Fig. 1).

In this review, we focus on (1) synthesis and distribution of OXT, (2) physiological functions of OXT, and (3) novel peptides, which stimulate OXT release (Table 1).

2. Synthesis and distribution of OXT

2.1. Regulation of synthesis and release of OXT

OXT is produced in the magnocellular neurosecretary cells of the SON and the PVN of the hypothalamus and is released into the systemic circulation from axon terminals in the neurohypophysis, particularly during parturition, lactation and in response to osmotic challenge [13]. The parvocellular OXT cells in the PVN, project their axon terminals to the brainstem and the spinal cord where OXT regulates autonomic functions [14]. Additional parvocellular OXT cells are found in the preoptic area and the lateral hypothalamus, whereas accessory magnocellular OXT cells are found scattered across the hypothalamus.

OXT is well known for its roles in reproduction, especially during and after childbirth. The pulsatile OXT release into the circulation is stimulated by vaginocervical stimulation associated with labor and suckling stimulus on the nipple. The uterine muscle increases its OXT receptor (OTR) and sensitivity to OXT during the latter few months of pregnancy. That level of OXT release from the neurohypophysis is considerably increased at the time of labor. In lactation, OXT causes milk to be expressed from the alveoli into the ducts of the breast that the baby can obtain it by suckling. The suckling stimulus on the nipple of the breast causes signals to be transmitted through sensory nerves to the OXT-secreting magnocellular neurons in the SON and the PVN. OXT in plasma is carried to the breast, where it causes contraction of myoepithelial cells that lie outside of and form a latticework surrounding the alveoli of the mammary glands. In less than a minute after baby's suckling, milk begins to flow.

OXT is also recognized as having endocrine and paracrine roles in male reproduction. OXT is synthesized within the mammalian testis, epididymis and prostate and OTRs in the reproductive tract supports a local action for OXT [15–23]. In ejaculation, a burst of OXT is released from the neurohypophysis into the systemic circulation and stimulates contractions of the reproductive tract for sperm release [24–26]. OXT has a paracrine role in stimulating contractility of the seminiferous tubules, epididymis and the prostate gland.

Interestingly, OXT is also released from soma and dendrites during parturition and lactation [27]. Although OXT released from soma and dendrites of magnocellular neurons in the SON and the PVN may act in a paracrine to activate distant receptors [27], OXT-like immunoreactivity (LI) fibers can be found throughout the brain, including the nucleus accumbens (NAcc), lateral septum, amygdala, and some areas in the hindbrain, brainstem, and spinal cord

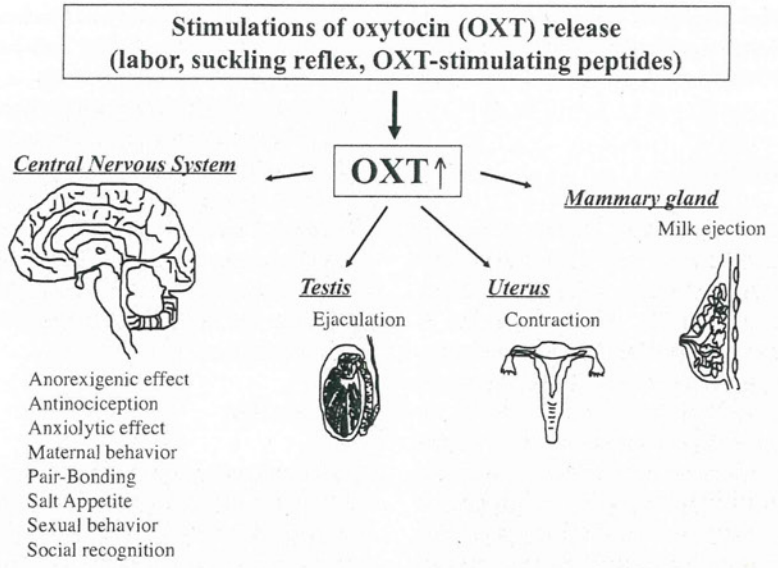

Fig. 1. Physiological function of OXT.

[14,28]. A notable reduction was observed in OXT-LI fibers throughout the brain by the lesioning the PVN [29]. Although little is known about the regulation of OXT release from these forebrain projections, they maybe contribute significantly to the regulation of behavior.

2.2. The OXT gene expression

The OXT gene expression is stimulated during pregnancy and lactation [30,31]. Interestingly, estrogen or progesterone alone does not increase OXT synthesis, however, the OXT gene expression in the SON and the PVN was increased by the administration of prolonged estrogen and progesterone, followed by progesterone withdrawal [32]. By contrast, the OXT gene expression in the uterine was highly stimulated by combined estrogen and progesterone application [33].

2.3. Distribution of OTR

The central effects of OXT are mediated by OTRs distributed widely in the brain. OTR mRNAs are distributed in the ventromedial nucleus of hypothalamus (VMH) and PVN which is involved in steroid-sensitive reproductive behaviors, in the PVN substantia nigra and ventral tegmental area which is involved in maternal behaviors, in the hippocampus which is involved in learning and memory and in

Table 1
Novel peptides which activate oxytocin (OXT) neurons and stimulate OXT release.

Peptides	Activation of oxytocin (OXT) neuron or OXT release		References
	OXT neuron	OXT release	
Adrenomedullin (AM)	Activate (icv)	Increase (icv)	[111,112]
Adrenomedullin 2 (AM2)/intermedin	Activate (icv)	Increase (icv)	[113,118]
Adrenomedullin 5 (AM5)	Activate (icv)	Increase (icv)	[120]
Apelin	Inhibite (icv)	Not determined (ND)	[136]
Cholecystokinin (CCK)	Activate (iv, ip)	Increase (iv, ip)	[138–141,145]
Cocaine- and amphetamine-regulated transcript (CART)	Activate (icv)	Increase	[76]
Corticotropin-released factor (CRF)	Not determined (ND)	Increase (icv, iv)	[220]
Kisspeptin	Activate (iv)	Increase (iv)	[173,175]
Glucagon-like Peptide-1 (GLP-1)	Activate (icv)	Increase (iv, high dose icv) Decrease (low dose icv)	[221–223]
α-melanocyte-stimulating hormone (αMSH)	Activate (icv)	Decrease (icv)	[224,225]
Nesfatin-1	Activate (icv)	Not determined (ND)	[181,182]
Neuromedin U (NMU)	Activate (icv)	Increase (icv)	[226]
Opioids (naloxone-induced OXT release)	Inhibite (icv)	Decrease (icv)	[227,228]
Prolactin-releasing peptide (PrRP)	Activate (icv)	Increase (icv)	[197,200]
Secretin	Activate (icv)	Increase (icv)	[215]
Thyrotropin-releasing hormone (TRH)	Not determined (ND)	Increase (iv)	[229]
Vasoactive intestinal polypeptide (VIP)	Not determined (ND)	Increase (icv)	[230]

the substantia nigra, ventral tegmental area, lateral septum, caudate putamen, amygdaloid nuclei, olfactory tubercle and cingulate, perirhinal, and frontal cortices which are involved in reinforcement [34].

2.4. The OTR gene expression

Gonadal steroids play an important role in mediating the regulation of the OTR expression. Most peripheral OXT-binding sites, including pituitary, renal, and uterine, are upregulated by estrogens [35–37]. The upregulation is accompanied by OTR mRNA expression. These results suggested that the upregulation is a consequence of a genomic estrogen effect on the OTR gene transcription [38,39]. In the behavioral studies clearly showed that a necessary potential of OXT to elicit maternal or sexual behavior is priming with estrogen alone or with both estrogen and progesterone [40,41]. This evidence suggests that OTR are under the control of gonadal steroids in the central nervous system (CNS).

The OTR gene expression increased during pregnancy and/or at parturition in olfactory bulb, medial preoptic area, bed nucleus of the stria terminalis (BNST), the SON, and the medial amygdala in rat [42,43]. OTRs-binding sites increased in the medial preoptic area, the BNST, VMH, and the ventral tegmental area on postpartum day 1 [42,44,45]. These changes suggest that OXT and OTR likely play a role in both lactation and the regulation of maternal behavior.

3. Pathophysiological function of OXT

3.1. Antinociception

OXT neurons in the PVN project not only to the posterior pituitary gland, but also to other brain areas and the spinal cord and OXT-LI fibers could be found in the dorsal horn of the spinal cord [46–48]. Several studies showed that OXT modulates nociception in the CNS. In the behavioral studies, intrathecally administered OXT enhanced antinociceptive effects in a dose-dependent manner in rats [47,49,50]. The antinociceptive effect, relief of low back pain, of intrathecally administered OXT was also reported in humans [51]. The antinociceptive effects of OXT were also shown in a model of experimental neuropathy developed following a spinal nerve ligation in rats [52]. The antinociceptive effects of intraperitoneally administered OXT were reversed by an OXT antagonist, not by naloxone [53]. After the administration of OXT into the periaquaductal gray, nucleus rapha magnus, and the NAcc, nociceptive response reduced in rats. These effects were blocked by OTRs antagonist [50,54]. As infusion of on opioid receptor antagonist dose-dependently attenuated the antinociceptive effects of OXT in the NAcc, the antinociceptive effects of OXT may involve an interaction with the opioid system [54]. Interestingly, recent study showed that OXT-induced analgesia was blocked in AVP receptor knockout mice [55]. The central and peripheral effects of OXT are thought to be mediated by its binding to a single isoform of the OTR [56], which activates the phospholipase Cβ (PLCβ) signal transduction pathway [57–59]. Centrally effects of AVP are mainly through the AVP-1A receptor (V1AR), which is also coupled to the PLCβ pathway [60–62]. Both OXT and AVP, as well as the OTR and V1AR, display a high degree of sequence homology, and both peptides can therefore activate both receptors [63]. OXT and AVP would have the functional interactions via their receptor system in antinociception.

3.2. Anxiety

Many studies reported that OXT had anxiolytic effects in rats, mice, and humans. In rats, intracerebroventricular (icv) administration of OXT suppressed the increase in plasma corticosterone level following 10 min of noise stress [64]. When rats placed in a novel environment, icv administration of OXT display decreased anxiety-like behavior in female rats in the elevated plus maze [65]. Moreover, OXT knockout mice display increasing anxiety-related behavior in the elevated plus maze, and enhanced corticosterone levels following a psychogenic stressor [66]. The effects of OXT on the stress response and on anxiety-like behavior are thought to partially mediate the anxiolytic effects observed during lactation. A steroid regimen that mimics changes in estradiol and progesterone during pregnancy, that is, estradiol and progesterone treatment followed by progesterone withdrawal, results in a decrease in anxiety-like behavior in the elevated plus maze and an attenuation of corticosterone secretion following noise stress in female rats. The effects of steroid treatment were blocked by OTR antagonist [67]. The treatment of estrogen has also been found to enhance the anxiolytic effects of OXT in mice [68]. The anxiolytic effects of estrogen may be mediated, at least in part, via OTR in the central nucleus of the amygdala. After the administration of OXT in the central nucleus of the amygdala (CeA), the anxiety-like behavior increase, while the administration of OTR antagonist into the CeA decreased anxiety-like behavior [69]. In human, intranasal OXT infusion enhanced the effects of social support in the suppression of cortisol secretion and subjective responses to psychosocial stress [70]. Moreover, functional magnetic resonance imaging study demonstrated that a similar intranasal OXT decreases activation at amygdala following the viewing of fear-inducing visual stimuli [71].

3.3. Feeding

OXT has an anorexic effect that is thought to play a role in signaling satiety. Icv administration of OXT inhibits feeding in hungry rats, while this inhibition of feeding was prevented by co-administration of OTR antagonist [72,73]. These studies were confirmed in the observations