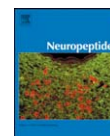


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The atypical antipsychotic, olanzapine, potentiates ghrelin-induced receptor signaling: an *in vitro* study with cells expressing cloned human growth hormone secretagogue receptor

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Abstract

The growth hormone secretagogue receptor (GHS-R) belongs to Gαq-coupled G protein-coupled receptor (GPCR) that mediates growth hormone release, food intake, appetite, glucose metabolism and body composition. Ghrelin has been identified as an endogenous ligand for GHS-R, and it is the only orexigenic peptide found in the peripheral organs. Olanzapine, an atypical antipsychotic agent that binds to and inhibits the activation of GPCR for several neurotransmitters, has metabolic side effects such as excessive appetite and weight gain. Recently, studies have revealed that the orexigenic mechanism of olanzapine is mediated via GHS-R signaling, although the precise mechanisms have not been clarified.

In this study, we investigated the effect of olanzapine on ghrelin-mediated GHS-R signaling by using an electrical impedance-based receptor biosensor assay system (CellKey™). Olanzapine at concentrations of 10^{-7} and 10^{-6} mol/L enhanced ghrelin-induced (10^{-10} – 10^{-8} mol/L) GHS-R activation. A Ca^{2+} imaging assay revealed that olanzapine (10^{-7} and 10^{-6} mol/L) enhanced ghrelin (10^{-7} M)-induced GHS-R activity. In contrast, haloperidol (an antipsychotic agent) failed to enhance this ghrelin-mediated GHS-R activation, as demonstrated by both the CellKey™ and Ca^{2+} imaging assays. Together, these results suggest that olanzapine, but not haloperidol, promotes appetite by enhancing ghrelin-mediated GHS-R signaling.

Key words: anorexia; appetite; Ca^{2+} imaging assay; CellKey™ system; ghrelin; growth hormone secretagogue receptor; haloperidol; olanzapine.

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1. Introduction

Growth hormone secretagogue receptor (GHS-R), whose endogenous ligand was identified to be ghrelin, has been proposed to mediate growth hormone release, food intake, appetite, glucose metabolism and body composition (Asakawa et al., 2003; Dezaki et al., 2004; Kojima et al., 1999; Pazos et al., 2008; Smith et al., 1997; Tolle et al., 2001; Yada et al., 2014). GHS-R belongs to G protein-coupled receptor (GPCR) that was first identified by Smith and colleagues in 1996 (Hill, 2006; Smith et al., 1997). Later, GHS-R was shown to be a Ca^{2+} -mobilizing, $\text{G}\alpha_q$ -coupled GPCR, and recently, it was recognized as a ghrelin receptor (Falls et al., 2006; Kojima et al., 1999). GHS-R is expressed in the hypothalamic-pituitary, ventral tegmental area, vagal afferent neurons, cardiovascular, immune, gastrointestinal, and reproductive systems and at high levels in the stomach and the arcuate nucleus of the hypothalamus (Cruz and Smith, 2008; Date et al., 2002; Fujino et al., 2003; Kojima et al., 1999; Yada et al., 2014).

Ghrelin, identified as an endogenous ligand for GHS-R by Kojima et al (Kojima et al., 1999), is a 28-amino acid peptide acylated with octanoic acid at the serine-3 position; the octanoic acid moiety is indispensable for ghrelin activity. Ghrelin plays an important role in triggering the adaptive response to starvation, and is the only orexigenic peptide in the peripheral organs, and it contributes to appetite reinforcement (Chen et al., 2009; Chen et al., 2010; Kojima et al., 1999). It is expressed at high levels in the stomach, where it regulates energy metabolism, promotes adiposity, and enhances appetite by activating GHS-R (Chen et al., 2009; Kojima et al., 1999). Previous clinical and preclinical studies have shown that ghrelin and ghrelin-mimetic GHS-R agonists activate

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GHS-R-mediated signaling and consequently, improve appetite, food intake, and body weight (Argiles and Stemmler, 2013; Chen et al., 2009; Costantini et al., 2011; Hassouna et al., 2013; Fujitsuka et al., 2011; Garcia et al., 2015; Moulin et al., 2013 ; Neary et al., 2004).

Olanzapine is an atypical antipsychotic drug that primarily employed to treat patients with schizophrenia (Fulton and Goa, 1997; Hartling et al., 2012). Olanzapine, which binds to several neurotransmitter receptors, including dopaminergic (D₁, D₂, D₃, and D₄), serotonergic (5HT_{2A} and 5HT_{2C}), histaminergic (H₁), and muscarinic (M₁, M₂, M₃, and M₄) receptors, has the undesirable side effects of promoting excessive appetite and weight gain (Bymaster et al., 1996; Domecq et al., 2015; Farwell et al., 2004; Leucht et al., 2009; Simon et al., 2009; (Selent et al., 2014; Stip and Lungu, 2015); Ujike et al., 2008). With taking advantage of those risks of olanzapine, it has been used to treat patients with anorexia nervosa and advanced cancer for promoting food intake and appetite (Bissada et al., 2008; F. Braiteh, 2008; Navari and Brenner, 2010; Stip and Lungu, 2015). However, the precise mechanisms by which olanzapine enhances appetite and weight gain are not understood. Previous reports suggested that the orexigenic effect of olanzapine is associated with ghrelin and GHS-R (Murashita et al., 2005; Naing et al., 2015; Sentissi et al., 2008; Zhang et al., 2013; Zhang et al., 2014).

Haloperidol is a typical antipsychotic drug that is widely employed to treat patients with schizophrenia (Hartling et al., 2012; Janssen et al., 1963). There have been few studies of haloperidol on the side effects such as excessive appetite and weight gain (Duggan et al., 2005; Leucht et al., 2009; Parsons et al., 2009).

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We hypothesized that the enhanced effects of olanzapine on weight and appetite are mediated by the augmentation of ghrelin signaling via enhancement of GHS-R activity. However, to the best of our knowledge, the effect of olanzapine on GHS-R activity has not been studied. In this study, we investigated whether olanzapine activates GHS-R-mediated signaling in cells expressing the human GHS-R. Data from previous studies on the orexigenic effects of olanzapine and haloperidol, we compared their effects on GHS-R-mediated signaling in cells expressing the human GHS-R.

2. Materials and Methods

2.1. Construction of plasmids and chemicals

Complementary DNA encoding human GHS-R was purchased from Kazusa DNA Research Institute (Chiba, Japan). Human Embryonic Kidney 293 subtype A cells (HEK293A cells) were obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA). HEK293A cells stably expressing human GHS-R were generated in our laboratory. The pN1-G-CAMP2 plasmid, encoding the intracellular calcium sensor G-CaMP2, was kindly gifted by Dr. J. Nakai (Saitama University Brain Science Institute, Saitama, Japan). G-CaMP2 is a fluorescent Ca^{2+} -sensing protein complex composed of mutated green-fluorescent protein, calmodulin (CaM), and Ca^{2+} /CaM-binding “M13” peptide (Mao et al., 2008; Nakai et al., 2001). The phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) immunoassay kit (Cat no. KCB1018) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Human ghrelin was obtained from Peptide Institute Inc. (Osaka, Japan), and was diluted with H_2O . Olanzapine and haloperidol were purchased from Wako Pure Chemical

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Industries, Ltd. (Tokyo, Japan), and were diluted in dimethyl sulfoxide (DMSO) for storage. All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture

HEK293A cells (expressing or not expressing human GHS-R) were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in a humidified atmosphere containing 95% air and 5% CO₂ at 37 °C (cell culture incubator).

2.3. Functional analysis of human GHS-R activation by ghrelin, olanzapine, and haloperidol with the CellKey™ system.

We measured GHS-R activity by using the electrical impedance-based biosensor, CellKey™ System (CellKey™), which was developed by MDS Analytical Technologies (Ontario, Canada). The CellKey™ assay utilizes cellular dielectric spectroscopy (CDS) in a label-free, real-time, kinetic cell-based approach, which enables comprehensive pharmacological evaluation of cells that exogenously or endogenously express receptors (Hisaoka-Nakashima et al., 2015; Miyano et al., 2014; Peters et al., 2010; Scott and Peters, 2010). The CellKey™ assay detects changes in the impedance of an induced extracellular current (dZ_{iec}), defined as the ratio of the voltage (applied by the CellKey™ instrument) to the current (measured by the CellKey™ instrument), as described by Ohm's law ($Z = V/I$; Miyano et al., 2014). In a previous study, a CellKey™ assay was performed on cells stably expressing GHS-R; the results revealed that ghrelin changed the cellular impedance in a concentration-dependent manner (Uezono et al., 2012).

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HEK293A cells (expressing or not expressing GHS-R) were seeded (density, 2.5×10^4 cells/well in DMEM) in CellKey™ 96-well microplates containing an electrode at the bottom of each well, and were incubated for 24 h (Miyano et al., 2014). After washing the wells with CellKey™ buffer composed of Hanks' balanced salt solution (1.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.81 mM MgSO_4 , 5.4 mM KCl, 0.44 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 136.9 mM NaCl, 0.34 mM Na_2HPO_4 , and 5.6 mM D-glucose) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1% bovine serum albumin, the cells were allowed to equilibrate in the CellKey™ buffer for 30 min before the assay.

Changes in dZiec were monitored by the CellKey™ assay system every 10 s for up to 20 min; a 5-min base line was recorded; the treatment drugs (ghrelin, olanzapine, and haloperidol) were added and dZiec was measured for 15 min subsequently. The extent of change in the dZiec values was expressed in terms of ΔZiec ($\Delta\text{Ziec} = \text{dZiec maximum} - \text{dZiec minimum}$) after drug application. The ratio of ΔZiec for each sample to that of the control sample (0.01% DMSO alone) was calculated.

We applied various concentrations of ghrelin, olanzapine, and haloperidol (diluted in CellKey™ buffer before use). First, ghrelin, olanzapine, haloperidol, or 0.01% DMSO was applied to HEK293A cells expressing GHS-R and mock-transfected cells (cells not expressing GHS-R but an empty vector). Second, olanzapine, haloperidol, or 0.01% DMSO was applied simultaneously with ghrelin to HEK293A cells expressing GHS-R. The concentrations of ghrelin, olanzapine, and haloperidol were chosen on the basis of the results of previous studies and their radio-receptor binding profiles (Bymaster et al., 1996; Fujitsuka et al., 2011; Uezono et al., 2012).

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2.4. Functional analysis of human GHS-R activation by ghrelin, olanzapine, and haloperidol with the Ca^{2+} imaging assay

Activation of Gαq-coupled receptors has been monitored by measuring increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of the cells of interest (Emkey and Rankl, 2009). The genetically encoded Ca^{2+} sensor, G-CaMP2, was used to visualize $[Ca^{2+}]_i$ (Fujitsuka et al., 2011; Mao et al., 2008; Nakai et al., 2001). HEK293A cells expressing GHS-R were seeded at a density of 2.5×10^5 cells/dish in glass-bottom, collagen-coated 35-mm culture dishes (WillCo Wells B.V., Amsterdam, Netherlands). Cells were transfected with G-CaMP2 using the X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Germany). After 24 h of transfection, the cells were washed twice with HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, pH 7.4). Ca^{2+} imaging was performed with a Meta 510 confocal microscope (Zeiss Japan, Tokyo, Japan). Fluorescence data were acquired and analyzed with the LSM510 META software (Carl Zeiss, Jena, Germany).

The concentrations of ghrelin, olanzapine, and haloperidol were chosen on the basis of the results of preliminary CellKey™ assays and previous studies including ours (Chuang et al., 2011; Fujitsuka et al., 2011; Uezono et al., 2012; Yada et al., 2012). Changes in $[Ca^{2+}]_i$ in response to ghrelin alone, and ghrelin together with olanzapine, haloperidol, or 0.01% DMSO diluted in HEPES buffer, were measured for 30 s. The results were expressed as the fluorescence intensity before and after test solution exposure. The fluorescence results were expressed as the ratio of the fluorescence intensity of each sample to that of the control sample (0.01% DMSO treated with ghrelin at the

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concentration used under each condition).

2.5. Assay of ERK1/2 activity following treatment with ghrelin and olanzapine/haloperidol in

HEK293A cells stably expressing GHS-R

HEK293A cells expressing GHS-R were cultured at a density of 2.0×10^4 cells/well in clear-bottomed 96-well microplates (included with the assay kit). After overnight culture in an incubator, the cells were washed twice with PBS and were exposed to olanzapine, haloperidol, or 0.01% DMSO with or without ghrelin. The concentrations were chosen on the basis of the results of preliminary CellKey™ assays and a previous study (M'Kadmi et al., 2015; Mousseaux et al., 2006). Five or 15 min after drug application, the treated cells were fixed with 8% formaldehyde in lysis buffer (R&D systems) to produce whole cell lysates. The whole cell lysates were exposed to lyophilized anti-phospho-specific extracellular signal-regulated protein kinases (ERK) 1/ERK2 (ERK1/2) antibodies and lyophilized anti-ERK1/2 antibodies, followed by exposure to horseradish peroxidase -conjugated goat anti-IgG secondary antibodies and alkaline phosphatase-conjugated goat anti-IgG secondary antibodies. Fluorogenic substrates for either horseradish peroxidase or alkaline phosphatase were added, and protein phosphorylation and total protein levels were measured simultaneously in each microplate well according to the instructions provided by the manufacturer of the phosphorylation of ERK1/2 (pERK1/2) immunoassay kit (Cat no. KCB1018; R&D Systems Inc., Minneapolis, MN, USA; Pierce et al., 2000). The ratio of pERK1/2 to total ERK1/2 was calculated for each treatment condition and was compared to the ratio obtained for cells treated with 0.01% DMSO (control) at each time point (in terms of % of control).

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2.6. Statistical analysis

All values are expressed as mean $\pm$ standard error of the mean (SEM). The non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's test was used to compare differences in ΔZ_{iec} following exposure to olanzapine, haloperidol or 0.01% DMSO(control) treated with ghrelin in the CellKeyTM assay, as well as to analyze changes in the Ca^{2+} imaging and *ERK1/2* activity assays. Non-parametric Friedman two-way ANOVA, followed by Bonferroni post-hoc analysis, was used to compare differences in ΔZ_{iec} following exposure to ghrelin, olanzapine, haloperidol, or 0.01% DMSO (control) alone in the CellKeyTM assay between HEK293A cells stably expressing GHS-R or mock-transfected cells. The threshold for statistical significance was $p < 0.05$. All analyses were performed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA).

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3. Results

3.1. Ghrelin increased ΔZ_{iec} in HEK293A cells expressing human GHS-R, as measured by the CellKey™ assay

We first applied ghrelin at a concentration range of 10^{-11} – 10^{-6} M to HEK293A cells stably expressing GHS-R or mock-transfected cells. As shown in Fig. 1, ghrelin increased ΔZ_{iec} in a concentration-dependent manner in HEK293A cells expressing GHS-R ($EC_{50} = 2.3 \times 10^{-8}$ M; Fig. 1). In contrast, there were no changes in ΔZ_{iec} in the mock-transfected cells (Fig. 1).

3.2. Olanzapine and haloperidol failed to increase ΔZ_{iec} in HEK293A cells regardless of the expression of human GHS-R

We next determined whether olanzapine and haloperidol by themselves activate GHS-R in HEK293A cells expressing GHS-R. As shown in Fig. 2, neither olanzapine nor haloperidol increased ΔZ_{iec} in HEK293A cells (regardless the expression of GHS-R) at any concentration tested.

3.3. Olanzapine but not haloperidol enhanced ghrelin-induced increases in ΔZ_{iec} in HEK293A cells expressing human GHS-R

The results shown in Figs. 1 and 2 demonstrated that ghrelin increased ΔZ_{iec} in HEK293A cells stably expressing GHS-R, as measured by the CellKey™ assay, and that neither olanzapine nor haloperidol alone increased ΔZ_{iec} in these cells. Next, we analyzed whether olanzapine or haloperidol, when applied together with ghrelin, could affect ghrelin-induced increases in ΔZ_{iec} in these cells. In this assay, ghrelin was applied at a concentration range of 10^{-8} – 10^{-6} M because previous studies and the current results have shown that ghrelin at these concentrations induces significant

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GHS-R activity (Fig.1; Uezono et al., 2012).

When olanzapine and ghrelin were simultaneously applied to the cells, olanzapine at concentrations greater than 10^{-7} M significantly enhanced ghrelin (10^{-10} M)-induced increases in ΔZ_{ie} ($p < 0.05$; Fig. 3A). Furthermore, exposure to olanzapine at 10^{-6} M enhanced ghrelin-induced increases in ΔZ_{ie} (10^{-9} – 10^{-8} M; $p < 0.05$; Figs. 3B, C).

In contrast, haloperidol at any concentration (10^{-8} – 10^{-6} M) did not enhance ghrelin-induced increases in ΔZ_{ie} (Figs. 4A, B, and C).

3.4. Olanzapine enhanced ghrelin-induced increase in intracellular $[Ca^{2+}]_i$ in HEK293A cells expressing human GHS-R

To clarify the mechanism through which olanzapine enhances ghrelin-induced GHS-R activity, we performed Ca^{2+} imaging assays with HEK293A cells stably expressing human GHS-R. As shown in Fig. 5A, ghrelin at concentrations of 10^{-11} – 10^{-5} M increased $[Ca^{2+}]_i$ in a concentration-dependent manner ($EC_{50} = 2.4 \times 10^{-7}$ M); this EC_{50} value was consistent with that estimated with the CellKey™ assay (Fig. 1). On the basis on these findings and the results of our previous studies (Fujitsuka et al., 2011; Uezono et al., 2012), we examined the effects of olanzapine and haloperidol on GHS-R-induced increases in $[Ca^{2+}]_i$ caused by treatment with ghrelin at a concentration of 10^{-7} M. When olanzapine (10^{-7} – 10^{-6} M), together with ghrelin, was applied to HEK293A cells expressing human GHS-R, olanzapine enhanced the ghrelin-elicited increase in $[Ca^{2+}]_i$ ($p < 0.05$; Fig. 5B). In contrast, haloperidol (10^{-7} and 10^{-6} M) failed to enhance the ghrelin-induced increase in $[Ca^{2+}]_i$ (Fig. 5C).

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3.5. Olanzapine did not enhance ghrelin-induced ERK1/2 activation in HEK293A cells expressing human GHS-R

GHS-R activation stimulates ERK, possibly via protein kinase C-mediated pathways (Pierce et al., 2000). Mousseau et al. demonstrated that activation of GHS-R by ghrelin is associated with increased pERK1/2 (Mousseaux et al., 2006). Therefore, we analyzed whether olanzapine enhances ghrelin-induced ERK1/2 activation in HEK293A cells expressing human GHS-R. pERK1/2 is a down-stream signaling molecule in ghrelin-mediated GHS-R signaling. We measured pERK1/2 abundance in HEK293A cells expressing human GHS-R, induced by the application of various concentrations of ghrelin, olanzapine, and haloperidol.

At 5 min after drug treatment, ghrelin at 10^{-7} M increased pERK1/2 abundance, compared with control; however, ghrelin at 10^{-8} M did not have this effect (control, 100 ± 3.9 ; ghrelin; 10^{-8} M and 10^{-7} M, 100.1 ± 5.8 and 173.8 ± 3.6 %, respectively; $p < 0.05$ [$n = 3$ each]). Olanzapine and haloperidol by themselves did not increase pERK1/2 abundance at any concentration (data not shown). Furthermore, neither olanzapine nor haloperidol enhanced ghrelin (10^{-7} M)-induced pERK abundance (ghrelin 10^{-7} M + 0.01% DMSO, 173.8 ± 3.6 ; ghrelin 10^{-7} M + olanzapine 10^{-6} M, 179.9 ± 8.4 ; ghrelin 10^{-7} M + haloperidol 10^{-7} M, 183.4 ± 13.8 %; $n = 3$ each).

At 15 min after drug treatment, ghrelin (10^{-8} – 10^{-7} M) did not increase pERK1/2 abundance compared with the control (control, 100 ± 4.4 ; ghrelin; 10^{-8} and 10^{-7} M, 108.7 ± 9.3 and 113.4 ± 9.0 %, respectively; $n = 3$ each). Neither olanzapine nor haloperidol increased pERK1/2 abundance (data not shown). Furthermore, both drugs failed to increase ghrelin (10^{-7} M)-mediated pERK1/2

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abundance (ghrelin 10^{-7} M + 0.01% DMSO, 113.4 ± 9.0 ; ghrelin 10^{-7} M + olanzapine 10^{-6} M, 97.5 ± 7.4 ; ghrelin 10^{-7} M + haloperidol 10^{-7} M, 131.0 ± 9.2 %; $p > 0.05$ [n = 3 each]).

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4. Discussion

The present study showed that olanzapine enhanced ghrelin-induced GHS-R signaling in HEK293A cells expressing human GHS-R. However, neither olanzapine nor haloperidol by itself activated GHS-R signaling, and haloperidol could not enhance ghrelin-induced GHS-R signaling. Our results suggest that olanzapine improves appetite through enhancement of ghrelin-induced GHS-R signaling.

In this study, we measured GHS-R activity by using an electrical impedance-based biosensor (the CellKey™ system) and the Ca^{2+} imaging assay. The CellKey™ system is specifically tailored to detect GPCR, and it produces response profiles that are consistent across a wide range of assay conditions. Moreover, the system can distinguish signals from different subtypes of G protein ($\text{G}\alpha\text{s}$, $\text{G}\alpha\text{i/o}$, and $\text{G}\alpha\text{q}$) as specific response profiles (Miyano et al., 2014; Scott and Peters, 2010). The agreement between the CellKey™ impedance assay for GPCR activation (including $\text{G}\alpha\text{q}$) and the traditional assays for GPCR signaling, such as cyclic adenosine monophosphate and $[\text{Ca}^{2+}]_i$ measurement, has been established previously including ours (Miyano et al., 2014; Peters et al., 2007; Scott and Peters, 2010). We previously demonstrated that GHS-R activation is associated with increased $[\text{Ca}^{2+}]_i$ in cells expressing GHS-R (Fujitsuka et al., 2011; Fujitsuka and Uezono, 2014). In this study, the CellKey™ system was used to measure the activity of the $\text{G}\alpha\text{q}$ -coupled GPCR in terms of increases in ΔZiec ; the assay generated specific response profiles of the $\text{G}\alpha\text{q}$ -coupled GPCR when olanzapine was applied with ghrelin to HEK293A cells expressing GHS-R (Fig.3). In addition, olanzapine alone failed to increase ΔZiec in HEK293A cells, regardless of GHS-R expression (Fig. 2).

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Therefore, we suggest that olanzapine enhances ghrelin-induced GHS-R signaling, although it inhibits the activation of other Gαq-coupled GPCR, including the H₁ receptor, type 2 serotonergic receptors, α-adrenergic receptor, and muscarinic receptors (Bymaster et al., 1996; (Mathews et al., 2012; Selent et al., 2014).

In this study, there are different concentrations of ghrelin effectiveness between the CellKey™ impedance assay (Fig. 3) and Ca²⁺ imaging assay (Fig. 5). Actually, ghrelin from 10⁻¹⁰ M increased dZiec in the CellKey™ assay (Fig 3), whereas ghrelin from 10⁻⁸ M increased Ca²⁺ in Ca²⁺ imaging assay (Fig 5). The possible reasons of such differences are based on their sensitivities of GPCR assay; first, the CellKey™ system detects total cellular responses different from the Ca²⁺ imaging assay for activation of Gαq-coupled GPCRs including GHS-R; second, the CellKey™ impedance assay are reported to be more sensitive for detecting GPCR activation than traditional assays such as Ca²⁺ imaging assay in previous studies (Miyano et al., 2014; Peters et al., 2007). Accordingly, we observed the dZiec increases by CellKey™ assay more efficiently than Ca²⁺ imaging assay for the activation of GHS-R, in the present study. We also showed that olanzapine enhanced the ghrelin-elicited increase in [Ca²⁺]_i in cells expressing GHS-R when applied together with ghrelin by using Ca²⁺-imaging assays. GHS-R mobilizes intracellular Ca²⁺; the activation of GHS-R by agonists such as ghrelin is coupled via Gαq-coupled GPCR to the phospholipase C pathway, leading to the formation of inositol triphosphate (IP) (Falls et al., 2006; Kaiya et al., 2014 ; Smith et al., 1997). Elevation of IP levels produces a subsequent increase in [Ca²⁺]_i from intracellular Ca²⁺ stores. In our previous study, the Japanese herbal medicine rikkunshito, as well as its constituent atracylodine, had no effect on [Ca²⁺]_i

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in cells expressing GHS-R alone; however, they robustly enhanced ghrelin-mediated signaling through augmentation of GHS-R-mediated increase in $[Ca^{2+}]_i$ (Fujitsuka et al., 2011). In the current study, we demonstrated that olanzapine, like rikkunshito, enhanced ghrelin-induced increases in $[Ca^{2+}]_i$ in cells expressing GHS-R by using Ca^{2+} -imaging assays, although the precise mechanism underlying this effect is unclear.

GHS-R is expressed in the hypothalamic-pituitary, ventral tegmental area, vagal afferent neurons, cardiovascular, immune, gastrointestinal, and reproductive systems and at a particularly high level in vagal afferent neurons in the stomach and in the hypothalamic arcuate nucleus (ARC) (Cruz and Smith, 2008; Date et al., 2002; Fujino et al., 2003; Kojima et al., 1999; Yada et al., 2014). Ghrelin enhances GHS-R signaling not only in the hypothalamic ARC, but also in the vagal afferent neurons in the stomach, sending vagal excitatory projections to neuropeptide Y (NPY) neurons (Date et al., 2002; Kageyama et al., 2010; Kohno et al., 2003). These mechanisms might be responsible for the effects of ghrelin on appetite (Date et al., 2002; Fujitsuka et al., 2011; Kageyama et al., 2010). In our previous study, rikkunshito augmented the ghrelin-induced increase in $[Ca^{2+}]_i$ in NPY neurons of the hypothalamic ARC, indicating that rikkunshito enhances ghrelin-induced cellular signaling in native NPY neurons in the ARC (Fujitsuka et al., 2011; Fujitsuka and Uezono, 2014; Kohno et al., 2003). Moreover, olanzapine upregulates the expression of NPY and the mRNA levels of GHS-R in the hypothalamic ARC (Ferno et al., 2011; Weston-Green et al., 2012; Zhang et al., 2013; Zhang et al., 2014). Therefore, olanzapine may enhance ghrelin-induced cellular signaling in the NPY neurons of the hypothalamic ARC.

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Some investigators have demonstrated that the activation of GHS-R also stimulates ERK, possibly via protein kinase C-mediated pathways (M'Kadmi et al., 2015; Mousseaux et al., 2006; Pierce et al., 2000). Our results demonstrated that the reinforcement of ghrelin-induced GHS-R signaling by olanzapine was related to changes in $[Ca^{2+}]_i$, but not to the activation of ERK1/2. Although $[Ca^{2+}]_i$ and pERK1/2 are down-stream pathways of GHS-R-mediated signaling, changes in $[Ca^{2+}]_i$ and pERK1/2 are mediated by different pathways (Emkey and Rankl, 2009; Pierce et al., 2000). In addition, no significant association has been detected between $[Ca^{2+}]_i$ changes and pERK1/2 abundance during the activation of GHS-R signaling (Mousseaux et al., 2006).

Recent clinical and preclinical studies have shown that ghrelin and ghrelin-mimetic GHS-R agonists enhance GHS-R-mediated signaling, suggesting that they can promote appetite and food intake (Argiles and Stemmler, 2013; Chen et al., 2009; Fujitsuka et al., 2011). In a clinical trial, ghrelin infusion significantly improved food intake, and the oral GHS-R agonist, anamorelin hydrochloride, promoted appetite and body weight (Garcia et al., 2015; Neary et al., 2004). In addition, some previous studies have demonstrated that ghrelin increases blood glucose, body weight and risk of diabetes, while GHS-R antagonists increase insulin secretion and decrease blood glucose, fat masses, body weight and food intake (Asakawa et al., 2003; Delhanty et al., 2012; Dezaki et al., 2004; Dezaki et al., 2006; Yada et al., 2014). In our previous *in vitro* and *in vivo* studies, rikkunshito improved appetite in rats, and when rikkunshito was applied together with ghrelin, it robustly enhanced ghrelin-induced increases in $[Ca^{2+}]_i$ in cells expressing GHS-R (Fujitsuka et al., 2011). In the current study, we demonstrated that olanzapine could enhance ghrelin-induced GHS-R signaling,

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by using both the CellKey™ impedance and Ca²⁺ imaging assays, only when applied together with ghrelin. Therefore, we hypothesize that olanzapine, similar to rikkunshito, may promote appetite by enhancing ghrelin-induced GHS-R signaling.

The precise mechanism of olanzapine's ability to promote appetite has not been understood. Some investigators have reported that the blockage of dopaminergic, serotonergic, and/or histaminergic receptors may increase appetite (He et al., 2014; Roerig et al., 2011). Other investigators have indicated that neuropeptide regulatory factors, including leptin and ghrelin, and/or a status of hormonal disturbance such as hyperprolactinemia and hypercortisolemia may be associated with food intake (Cummings et al., 2002; Davey et al., 2012; Esen-Danaci et al., 2008; Murashita et al., 2005; Perez-Iglesias et al., 2008; Rojczyk et al., 2015; Zhang et al., 2013). In these studies, olanzapine either upregulated or downregulated circulating serum ghrelin levels in humans and animals (Esen-Danaci et al., 2008; Lu et al., 2015; Murashita et al., 2005; Naing et al., 2015). We previously demonstrated that rikkunshito ameliorates ghrelin resistance by reinforcing ghrelin-induced GHS-R activation (Fujitsuka et al., 2011; Terawaki et al., 2015). In addition, in a study by Zhang et al., olanzapine-treated rats showed hyperphagia and weight gain; administration of a GHS-R antagonist reversed these effects (Zhang et al., 2014). Therefore, we suggest that olanzapine improves appetite by reinforcing ghrelin-induced GHS-R activity and ameliorating ghrelin resistance in GHS-R rather than by enhancing circulating ghrelin levels.

In the present study, the concentrations of olanzapine used in the experiments were similar to the serum concentrations observed upon administration of the clinical dose (serum concentration after

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12 h, 25–150 nM; Callaghan et al., 1999; Olesen and Linnet, 1999). Therefore, the concentrations of olanzapine used in our study are similar to the concentrations that promote appetite at clinical doses. Olanzapine affects for homeostatic mechanisms including endocrine system and brain reward circuitry including several neurotransmitter receptors. It occasionally induces side effects such as sedation, akathisia and hyperglycemia (Chakos et al., 2001; Domecq et al., 2015; Mathews et al., 2012; Ober et al., 1999). Some clinical trials have demonstrated the safety of olanzapine administration at clinical doses; even advanced cancer patients can take olanzapine (Bissada et al., 2008; Fulton and Goa, 1997; Navari et al., 2011; Passik et al., 2002). We also hypothesized that olanzapine may cause glycaemia and diabetes by the enhancement of ghrelin signaling, which restricts insulin release and upwardly regulates the systemic glucose level (Dezaki et al., 2004; Dezaki et al., 2006; Yada et al., 2014).

Olanzapine has been used to promote non-fluid weight gain and appetite in advanced cancer patients with cachexia (F. Braithe, 2008; Naing et al., 2015; Navari and Brenner, 2010). In addition, some clinical studies have shown that ghrelin and ghrelin-mimetic GHS-R agonists may promote appetite and improve cancer-related cachexia (Argiles and Stemmler, 2013; Garcia et al., 2015; Neary et al., 2004). Symptoms of cachexia often occur in advanced cancer patients, leading to physical pain, emotional pain, and social distress, while significantly decreasing the quality of life and survival for cancer patients (Andreyev et al., 1998; Blum et al., 2014; Fearon et al., 2011; Strasser and Bruera, 2002). We previously reported that rikkunshito promoted appetite and improved cancer-related anorexia and cachexia by ameliorating ghrelin resistance through enhancement of ghrelin-induced

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GHS-R signaling in mice (Fujitsuka et al., 2011). Similarly, enhancement of ghrelin-induced GHS-R signaling by olanzapine may promote appetite and improve cachexia in advanced cancer patients.

Our study demonstrated that haloperidol failed to reinforce ghrelin-induced GHS-R activation. In the clinical setting, haloperidol does not promote excessive appetite and weight gain (Leucht et al., 2009; Parsons et al., 2009). These differences on promoting appetite between olanzapine and haloperidol may be attributed to their distinct effects on ghrelin-induced GHS-R signaling.

These results suggest that olanzapine may promote appetite by enhancing ghrelin-induced GHS-R signaling, while olanzapine inhibits the activation of other $G\alpha_q$ -coupled GPCR. In our previous study, the orexigenic Japanese herbal medicine rikkunshiro directly enhances the ghrelin-induced GHS-R, while it inhibits 5-HT_{2B} and 5HT_{2C} receptors (Fujitsuka et al., 2011; Fujitsuka and Uezono, 2014; Takeda et al., 2008; Uezono et al., 2012). The researchers found five active compounds that modulate the signaling of GPCRs in rikkunshito; atractylodin, atractylodinol, hesperidin, isoliquiritigenin and heptamethoxyflavone (Fujitsuka et al., 2011; Takeda et al., 2008). Atractylodin and atractylodinol showed a marked increase in ghrelin-induced GHS-R activity (Fujitsuka et al., 2011; Uezono et al., 2012). On the other hand, hesperidin, isoliquiritigenin and heptamethoxyflavone inhibit both 5-HT_{2B} and 5HT_{2C} receptor (Takeda et al., 2008; Uezono et al., 2012). Therefore, we suppose that mechanisms of olanzapine in enhancing GHS-R while suppressing other GPCRs are similar to the effects of ingredients in rikkunshito, although olanzapine was made of single chemical. Based on these data, we may suggest the possible mechanisms of olanzapine in

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enhancing ghrelin-induced GHS-R signaling are similar to atractylodin and atractylodinol in the rikkunshito (Fujitsuka et al., 2011; Fujitsuka and Uezono, 2014; Uezono et al., 2012).

5. Conclusion

The present study showed that olanzapine enhances GHS-R signaling in cells expressing human GHS-R; however, olanzapine by itself has no effect on GHS-R activation. This finding indicates that olanzapine promotes appetite by enhancing ghrelin-induced GHS-R signaling. The results of the present study are limited to *in vitro* conditions. Further studies are needed to elucidate the comprehensive mechanism of action of olanzapine in ghrelin-mediated signaling.

Conflicts of interest

The authors have no conflicts of interest to report.

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Figure Captions

Figure 1. Effects of ghrelin (in terms of ΔZ_{iec}) in Human Embryonic Kidney 293A (HEK293A) cells expressing human GHS-R, as measured by the CellKey™ system.

Changes in ΔZ_{iec} induced by ghrelin at concentrations of 10^{-11} – 10^{-6} M in HEK293A cells stably expressing human GHS-R (●) or mock-transfected cells (□). All data are presented as the mean $\pm$ standard error of the mean (SEM) for each condition ($n = 5$ – 18). Ghrelin increased ΔZ_{iec} in a concentration-dependent manner; significant differences were observed at 10^{-8} – 10^{-6} M of ghrelin between cells expressing and not expressing GHS-R (non-parametric with Friedman two-way ANOVA followed by Bonferroni post-hoc analysis; *** $p < 0.001$; n.s., not significant; $p > 0.05$). ANOVA, analysis of variance; DMSO, 0.01% dimethyl sulfoxide vehicle; ΔZ_{iec} , extent of change in the impedance (maximum–minimum) of an induced extracellular current, as measured by the CellKey™ system; GHS-R, growth hormone secretagogue receptor; M, mol/L.

Figure 2. Effects of olanzapine and haloperidol on ΔZ_{iec} in HEK293A cells expressing human GHS-R.

Neither olanzapine nor haloperidol induced changes in ΔZ_{iec} in HEK293A cells expressing (●) or not expressing (□) GHS-R, as shown in A (olanzapine [10^{-8} – 10^{-6} M]) and B (haloperidol [10^{-8} – 10^{-6} M]). All data are presented as the mean $\pm$ SEM at each condition ($n = 3$ – 12). No significant differences were observed at each condition between cells expressing and not expressing GHS-R (non-parametric with Friedman two-way ANOVA followed by Bonferroni post-hoc analysis; n.s., not

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significant, $p > 0.05$). ANOVA, analysis of variance; DMSO, 0.01% dimethyl sulfoxide vehicle;

ΔZ_{iec} , extent of change in the impedance (maximum–minimum) of an induced extracellular current,

as measured by the CellKey™ system; GHS-R, growth hormone secretagogue receptor; M, mol/L.

Figure 3. Olanzapine enhanced ghrelin-induced increases in ΔZ_{iec} in HEK293A cells expressing human GHS-R.

Olanzapine enhanced ghrelin-induced increases in ΔZ_{iec} in HEK293A cells expressing human

GHS-R. A) Olanzapine (10^{-8} – 10^{-6} M) with 10^{-10} M ghrelin; B) olanzapine (10^{-8} – 10^{-6} M) with 10^{-9} M

ghrelin; and C) olanzapine (10^{-8} – 10^{-6} M) with 10^{-8} M ghrelin. All data are presented as the mean $\pm$

SEM of 4 independent experiments ($n = 6$ – 12), as measured with the CellKey™ assay system.

Olanzapine at concentrations of 10^{-7} M and 10^{-6} M significantly enhanced ghrelin-induced increases

in ΔZ_{iec} compared with the control (DMSO plus ghrelin; non-parametric with Kruskal-Wallis

one-way ANOVA followed by Dunn's test; * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$; n.s., not significant,

$p > 0.05$). ANOVA, analysis of variance; DMSO, 0.01% dimethyl sulfoxide vehicle; ΔZ_{iec} , change in

the impedance of an induced extracellular current, as measured by the CellKey™ system; ΔZ_{iec} ,

extent of change in the impedance (maximum–minimum) of an induced extracellular current, as

measured by the CellKey™ system; GHS-R, growth hormone secretagogue receptor; M, mol/L.

Figure 4. Haloperidol failed to enhance ghrelin-induced increases in ΔZ_{iec} in HEK293A cells expressing GHS-R.

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Haloperidol did not enhance ghrelin-induced increases in ΔZ_{iec} in HEK293A cells expressing human GHS-R. A) Haloperidol (10^{-8} – 10^{-6} M) and 10^{-10} M ghrelin; B) haloperidol (10^{-8} – 10^{-6} M) and 10^{-9} M ghrelin; and C) haloperidol (10^{-8} – 10^{-6} M) and 10^{-8} M ghrelin. All data are presented the mean $\pm$ SEM of 3 independent experiments (n = 3–9) performed using the CellKey™ assay system (non-parametric with Kruskal-Wallis one-way ANOVA followed by Dunn's test; n.s., not significant, $p > 0.05$). ANOVA, analysis of variance; DMSO, 0.01% dimethyl sulfoxide vehicle; ΔZ_{iec} , extent of change in the impedance (maximum–minimum) of an induced extracellular current, as measured by the CellKey™ system; GHS-R, growth hormone secretagogue receptor; M, mol/L.

Figure 5. Olanzapine but not haloperidol enhanced ghrelin-induced increases in $[\text{Ca}^{2+}]_i$ in HEK 293A cells expressing human GHS-R.

Ghrelin increased $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner, and olanzapine, but not haloperidol, enhanced the ghrelin-induced increases in $[\text{Ca}^{2+}]_i$ in HEK293A cells expressing human GHS-R, as determined by Ca^{2+} imaging assays. A) Ghrelin (10^{-10} – 10^{-6} M) alone (n = 6–12); B) olanzapine (10^{-7} – 10^{-6} M) with 10^{-7} M ghrelin (n = 61–87); and C) haloperidol (10^{-7} – 10^{-6} M) with 10^{-7} M ghrelin (n = 11–23). All data are presented as the mean $\pm$ SEM at each condition (non-parametric with Kruskal-Wallis one-way ANOVA followed by Dunn's test; $*0.01 \leq p < 0.05$, $**0.001 \leq p < 0.01$, $***p < 0.001$; n.s., not significant, $p > 0.05$). ANOVA, analysis of variance; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; DMSO, 0.01% dimethyl sulfoxide vehicle; GHS-R, growth hormone secretagogue receptor; M, mol/L.

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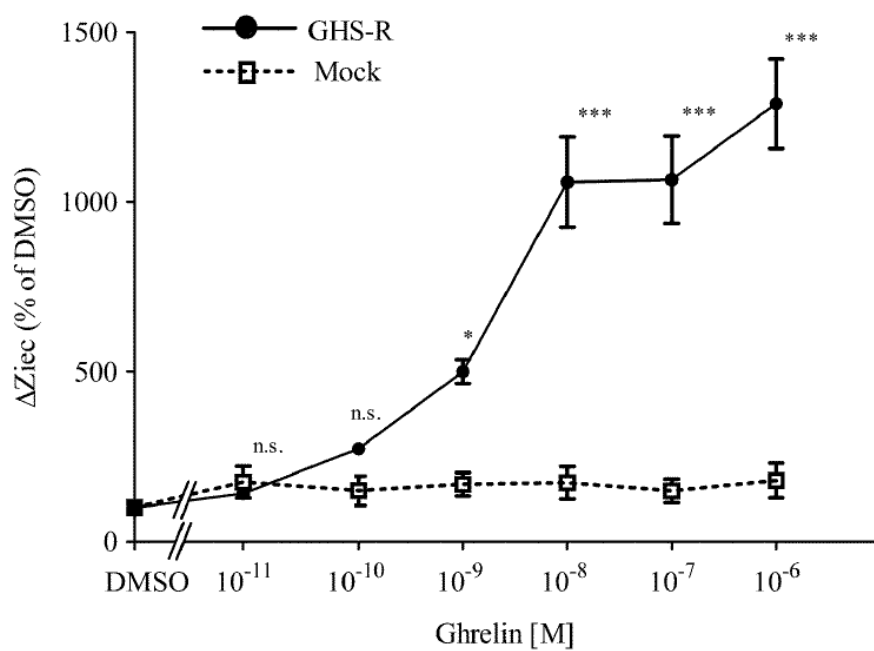


Fig.1

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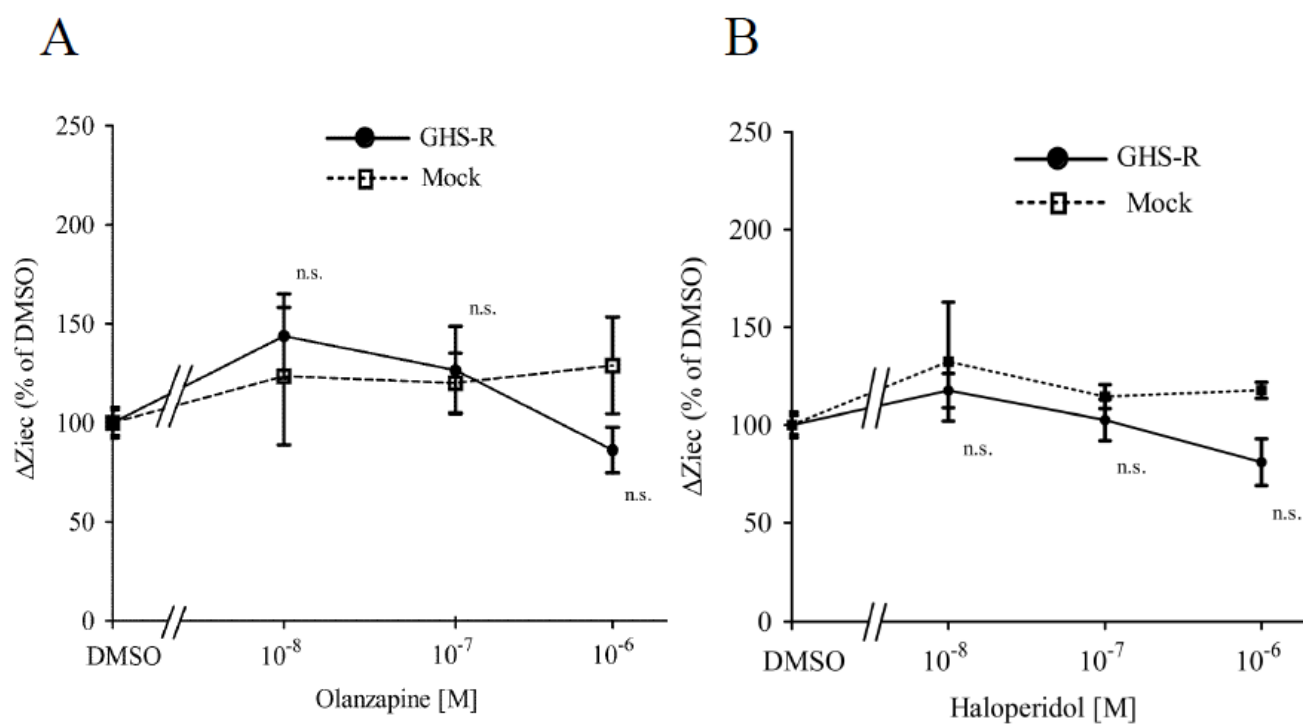


Fig.2

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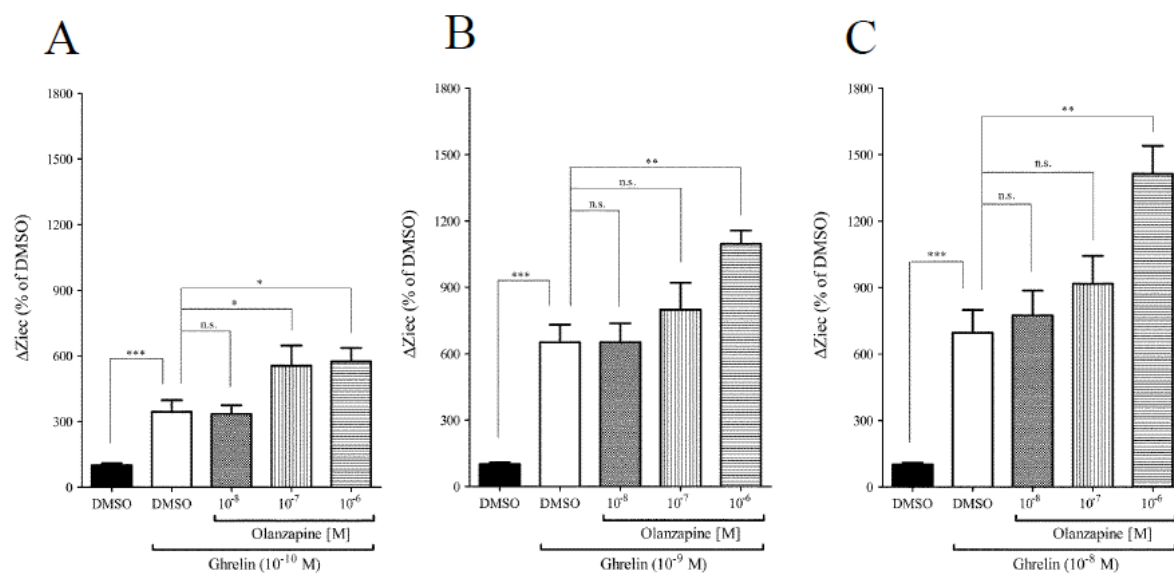


Fig.3

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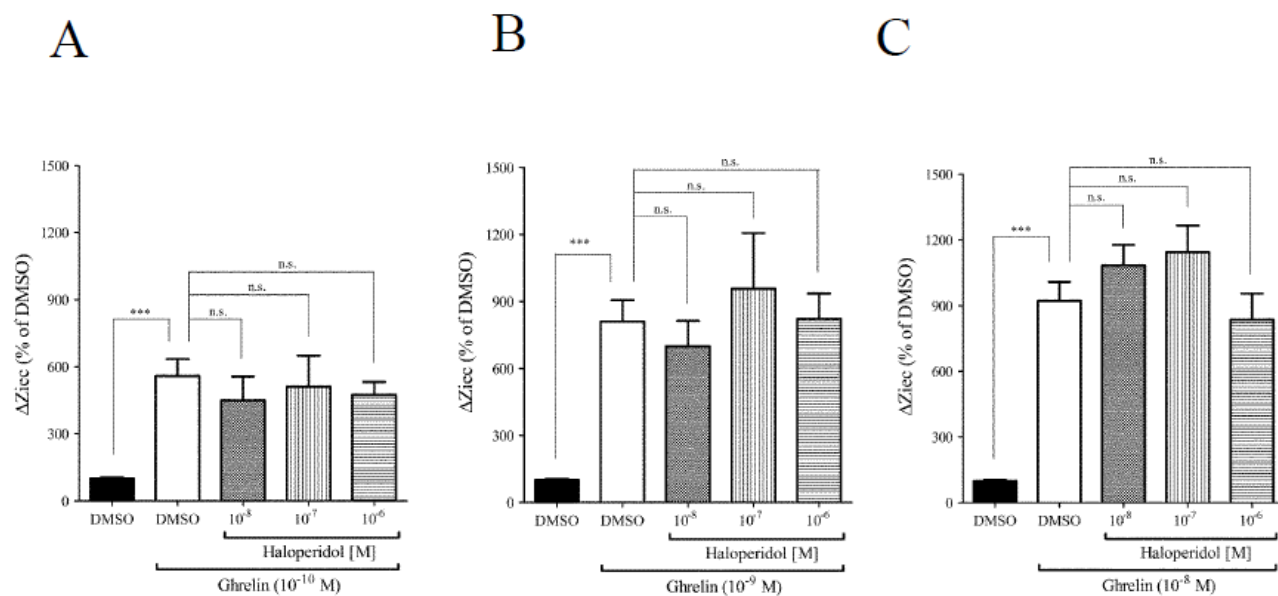


Fig.4

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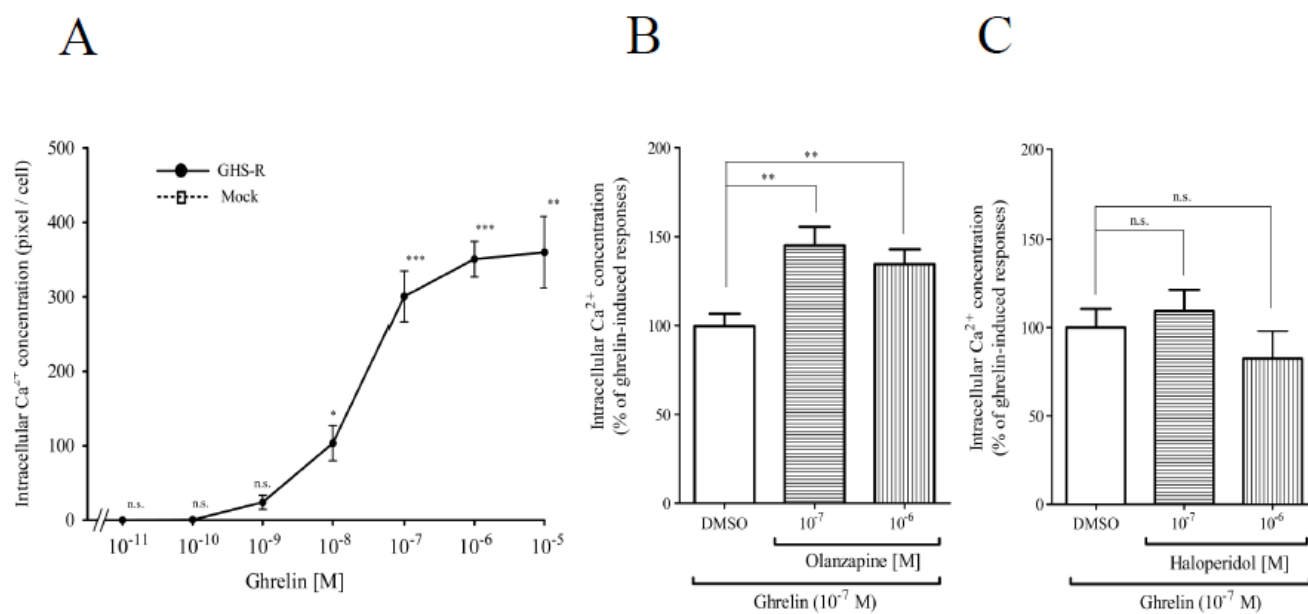


Fig.5

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Highlights:

- The orexigenic mechanism of olanzapine has not been clarified.
- We examined the association between this effect and ghrelin receptor signaling.
- We examined this relationship by using cellular impedance and Ca^{2+} imaging assays.
- Olanzapine enhanced the ghrelin receptor-mediated signaling induced by ghrelin.
- Olanzapine may promote appetite by enhancing ghrelin receptor signaling.