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Ghrelin's orexigenic effect is modulated via a serotonin 2C receptor interaction *

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*Running Title:

GHS-R1a receptor-induced food intake is altered following 5-HT$_{2C}$ receptor modulation

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Key words

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Abstract

Understanding the intricate pathways modulating appetite and subsequent food intake is of particular importance considering the rise in obesity incidence across the globe. The serotonergic system, specifically the 5-HT_{2C} receptor, has shown to be of critical importance in the regulation of appetite and satiety. The GHS-R1a receptor is another key receptor well-known for its role in the homeostatic control of food intake and energy balance. We recently showed compelling evidence for an interaction between the GHS-R1a receptor and the 5-HT_{2C} receptor in an *in vitro* cell line system heterologously expressing both receptors. Here, we investigated this interaction further. First, we show that the GHS-R1a/5-HT_{2C} dimer-induced attenuation of calcium signalling is not due to coupling to $G\alpha_S$, as no increase in cAMP signalling is observed. Next, flowcytometry fluorescence resonance energy transfer (fcFRET) is used to further demonstrate the direct interaction between the GHS-R1a receptor and 5-HT_{2C} receptor. In addition, we demonstrate co-localized expression of the 5-HT_{2C} and GHS-R1a receptor in cultured primary hypothalamic- and hippocampal rat neurons, supporting the biological relevance of a physiological interaction. Furthermore, we demonstrate that when 5-HT_{2C} receptor signalling is blocked, ghrelin's orexigenic effect is potentiated *in vivo*. In contrast, the specific 5-HT_{2C} receptor agonist lorcaserin, recently approved for the treatment of obesity, attenuates ghrelin-induced food intake. This underscores the biological significance of our *in vitro* findings of 5-HT_{2C} receptor-mediated attenuation of GHS-R1a receptor activity. Together, this study demonstrates, for the first time, that the GHS-R1a/5-HT_{2C} receptor interaction translates into biological significant modulation of ghrelin's orexigenic effect. This data highlights the potential development of a combined GHS-R1a and 5-HT_{2C} receptor treatment strategy in weight management.

Introduction

The gastric-derived-peptide ghrelin acts as the endogenous ligand for the growth hormone secretagogue (GHS-R1a) receptor, which is also known as the ghrelin receptor [1, 2]. Ghrelin is the only known gut-peptide exerting an orexigenic effect via the activation of the centrally expressed GHS-R1a receptor [3-6] and has thus received much attention as an anti-obesity drug target [7-16]. However, despite previous and ongoing drug development efforts, no weight-loss drugs that target the ghrelin receptor are currently on the market.

Initially, the GHS-R1a receptor was found to function as a homodimer [17, 18]. However, recently, the GHS-R1a receptor has also been shown to heterodimerize with other GPCRs involved in appetite regulation and food reward (for review see [19]), including its truncated splice variant, the GHS-R1b receptor [18, 20-22], the melanocortin 3 receptor (MC_3) and the dopamine receptors (D_1 and D_2) [23-27]. Moreover, our lab has demonstrated compelling evidence for a functional interaction between the GHS-R1a and the 5-HT_{2C} receptor [27].

Interestingly, serotonergic signaling has since long been known to be involved in controlling food intake and to impact on satiety [28-38]. Individuals with normal regulated brain serotonin (5-hydroxytryptamine, 5-HT) levels are more easily satiated and display a better control over carbohydrate cravings inhibiting sugar intake more readily [39, 40]. Moreover, several drugs targeting the central serotonergic system, such as sibutramine and fenfluramine, have been specifically developed to induce satiety, or have been found to reduce food intake as a secondary effect, such as is the case for the 5-$HT_{2B/2C}$ agonist m-chlorophenylpiperazine (mCPP) [29, 37, 41, 42]. Unfortunately, none of these drugs have been without heart and pulmonary vasculature side-effects, or have been associated with a poor efficacy and other non-specific effects [33].

The centrally expressed serotonin 2C (5-HT_{2C}) receptor, in particular, has been shown to stimulate satiety via excitatory neurotransmission [29-34, 43]. Indeed, a large amount of literature has validated the critical role played by the 5-HT_{2C} receptor, which has substantiated this receptor as a viable target for the development of therapeutics in appetite control and weight

management [30-38]. The recently approved 5-HT_{2C} agonist lorcaserin is the first successful 5-HT_{2C} receptor-targeting drug to reduce weight in the treatment of obesity [44-47].

Interestingly, the expression of the central 5-HT_{2C} receptor [48, 49] corresponds with the expression profile of the neuronal circuits expressing the GHS-R1a receptor [50-52], which is a first requirement for a physical interaction or dimerization. In addition, reciprocal interactions between the serotonin and ghrelin signalling pathways have been described previously. Indeed, administration of ghrelin to hypothalamic synaptosomes [53] was shown to inhibit 5-HT release, as was direct administration of ghrelin to hippocampal slices [54]. Similarly, recent data has demonstrated an increased serotonergic turnover in the amygdala and altered serotonin receptor mRNA levels (including the 5-HT_{2C} receptor) in the amygdala and dorsal raphe, following acute central ghrelin administration [55]. Moreover, attenuated increases in acylated-ghrelin were observed in response to an overnight fast in mice following pharmacological increases of brain serotonin levels or direct 5-HT_{2C} receptor agonism [56]. In addition, direct administration of serotonin or the 5-HT_2 receptor agonist 5-dimethoxy-4-iodoamphetamine (DOI) attenuated ghrelin's orexigenic effect in rats [57]. We hypothesize that this serotonin-mediated attenuation of ghrelin signalling is mediated via crosstalk of the GHS-R1a receptor with the 5-HT_{2C} receptor, potentially in a direct physical interaction. In line with this hypothesis, we have previously shown a functional interaction between the GHS-R1a and 5-HT_{2C} receptor *in vitro* [27], demonstrating an attenuated GHS-R1a signalling following co-expression of the 5-HT_{2C} receptor, which reinforces the physiological relevance of the GHS-R1a/5-HT_{2C} dimer.

However, although evidence for dimerization *in vitro* is compelling, in general the existence of GPCR dimers in native tissue has been questioned because of the paucity of reports demonstrating an interaction *in vivo*. In this study, we further investigate the interaction between the GHS-R1a and 5-HT_{2C} receptor in relation to its function in appetite and we analyse the significance of the interaction of these two key receptors *in vivo*. Specifically, the co-localized expression of endogenous levels of these receptors in neuronal cultures is investigated using a recently described fluorescein-labelled ghrelin peptide tracer [58, 59]. Finally, the effects of specific

5-HT_{2C} receptor antagonism versus agonism on ghrelin's orexigenic effect is analysed in mice. To our knowledge this is the first study to show functional relevance of a specific GHS-R1a and 5-HT_{2C} receptor interaction on food intake behaviour *in vivo*. This data suggest the potential of combined GHS-R1a receptor antagonism and 5-HT_{2C} receptor agonism as a novel therapeutic strategy in weight management.

Results and Discussion

Fluorescence energy transfer upon co-expression of the GHS-R1a receptor with the 5-HT_{2C} receptor

Heterodimerization of the GHS-R1a receptor with two variants of the 5-HT_{2C} receptor was investigated using flow cytometry fluorescence energy transfer (FRET). To this end, Hek293A cells stably expressing the unedited 5-HT_{2C} receptor or a partially edited isoform, 5-HT_{2C}-VSV-eGFP, both c-terminally fused with an enhanced green fluorescent fusion protein (eGFP), were transduced with lentiviral vectors expressing the GHS-R1a receptor C-terminally fused with a red fluorescent tag (lvGHS-R1a-TagRFP). The 5-HT_{2C} receptor is prone to post-transcriptional RNA editing, which is the enzymatic conversion of an adenosine to inosine residues on 5 specific nucleotide positions (A, B, C, D, E) in the 2nd intracellular loop and is thought to be associated with a reduced receptor functioning [60-66]. Therefore, we included both the unedited 5-HT_{2C} receptor and the partly edited 5-HT_{2C}–VSV receptor, which is the most abundantly expressed 5-HT_{2C} receptor isoform in human brain. Indeed, the 5-HT_{2C}–VSV receptor isoform is particularly abundant in the hypothalamus [65, 67], where an increased 5-HT_{2C} receptor editing has been linked with changes in feeding behaviour and fat mass [38, 66, 68]. Noteworthy increases in FRET levels, as percentage of tagRFP expression, were observed 72hrs post transduction (Figure 1). Following lentiviral transduction of Hek293 cells with the lvGHS-R1a-tagRFP vector, 61.6% of cells were analysed as positive for tagRFP expression (Figure 1, 1st row, column 2), with relatively no FRET signal (1.6%, Figure 1, 2nd row, column 2), which demonstrates successful lentiviral transduction. In addition, no tagRFP or FRET signal was observed in Hek293 wild type (Hek293

wt) cells or Hek293 cells stably expressing 5-HT_{2C}-eGFP or the 5-HT_{2C}-VSV variant (Figure 1, 1st and 2nd row). Hek cells stably expressing 5-HT_{2C}-eGFP or the 5-HT_{2C}-VSV variant showed an increase in tagRFP expression of respectively 38.7% and 61.5%, when transduced with the control-tagRFP vector (Figure 1, 3rd row, column 1 and 2). Similar percentages of 68.3% and 52.2% were observed following transduction with the lvGHS-R1a-tagRFP vector in Hek 5-HT_{2C}-eGFP or the Hek 5-HT_{2C}-VSV-eGFP cells, respectively (Figure 1, 3rd row, column 3 and 4). Finally, when analysing flow cytometry fluorescence energy transfer as a measure of heterodimerisation, co-expression of GHS-R1a-tagRFP in Hek293 5-HT_{2C}-eGFP or Hek293 5-HT_{2C}-VSV-eGFP cells increased FRET signal from 1.2% to 12.8% and 1.9% to 30.26% compared to control-TagRFP vectors, respectively (Figure 1, 4th row). These significant increases in FRET signal are further evidence of a physical interaction between the GHS-R1a receptor and the 5-HT_{2C} receptor. Interestingly, we consistently found a >2x higher percentage of FRET signal when the GHS-R1a receptor is co-expressed with the edited 5-HT_{2C}-VSV variant of the receptor compared to the fully unedited 5-HT_{2C} receptor. This may suggest that 5-HT_{2C} receptor editing can modulate dimer formation and warrants further investigations.

Co-expression of the 5-HT_{2C} receptor attenuates GHS-R1a-mediated intracellular calcium mobilization without altering cAMP signalling

The GHS-R1a receptor as well as the 5-HT_{2C} receptor couple to the Gq protein, which leads to Gq-subunit mediated increase in phospholipase C, which subsequently elevates intracellular calcium levels. To assess the functional consequences of an interaction of the GHS-R1a receptor with the 5-HT_{2C} receptor we analysed ligand-mediated downstream signalling consequences following co-expression of fluorescently tagged receptors. To this end, heterologous cells co-expressing the GHS-R1a-EGFP receptor and the 5-HT_{2C}-RFP receptor were analysed for ligand-mediated intracellular calcium increase as well as intracellular cAMP levels. The dose-dependent ghrelin-mediated intracellular calcium influx in Hek293 cells stably expressing the GHS-R1a receptor, previously shown to be independent of fluorescent tag (Schellekens, van Oeffelen et al. 2013), was reduced when co-expressing the 5-HT_{2C} receptor (Figure 2 A). In

addition, a similar attenuation of the GHS-R1a-mediated intracellular calcium mobilization upon co-expression of the 5-HT_{2C} receptor was observed when the synthetic GHS-R1a ligand, MK0677, was used (Figure 2 B). This is in line with our previous study and confirms the 5-HT_{2C} receptor-mediated attenuation of GHS-R1a receptor signalling, which concurs the interaction between the two receptors [27]. Previously, it has been shown that the GHS-R1a receptor dimerizes with the dopamine D_1 receptor leading to an enhanced dopamine induced c-AMP accumulation [24] and an attenuation of GHS-R1a-mediated calcium signalling [27]. This may suggest a dimer-induced switch in GHS-R1a receptor G-protein coupling from $G\alpha_q$ to $G\alpha_s$, which has been previously suggested for neuronal GHS-R1a receptors expressed in neuropeptide Y (NPY) cells of the arcuate nucleus of the hypothalamus [69]. Thus, we set out to determine if the attenuated GHS-R1a receptor-mediated calcium mobilization observed here is due to a switch in G protein coupling from $G\alpha_q$ to $G\alpha_s$. To this end, we measured cAMP increases in Hek293 cells expressing single receptors or co-expressing both the GHS-R1a and 5-HT_{2C} receptors (Figure 3). First, we analysed Hek293 cells transduced with the D_1 receptor expressing vectors (lvDRD1-tagRFP) as a positive controls (Figure 3A and B), as the D_1 receptor is coupled to the G protein $G\alpha_s$, and receptor ligand binding subsequently activates adenylyl cyclase, leading to increasing intracellular concentrations of the second messenger cAMP. Indeed, a significant increase in intracellular cAMP was observed following exposure to the D_1 agonist, 6,7-ADTN hydrobromide (0.5nM), in Hek293 cells transiently expressing the D_1 receptor following lentiviral transduction but not in cells stably expressing the 5-HT_{2C} receptor (Figure 3A) or the GHS-R1a receptor (Figure 3B). No cAMP responses were observed in Hek293 cells transiently expressing the D_1 receptor following serotonin (100nM) or ghrelin (100nM) exposure (Figure 3A and B). In addition, no ligand-mediated cAMP responses were observed in Hek293 cells stably expressing the 5-HT_{2C}-eGFP receptor (Figure 3A and 3C) or in 5-HT_{2C}-expressing cells transduced with lv-GHS-R1a-tagRFP vectors (Figure 3C). Moreover, no ligand-mediated cAMP response were observed in Hek-GHS-R1a-EGFP cells (Figure 3B and D) or in Hek-GHS-R1a-EGFP cells lentivirally transduced to express 5-HT_{2C}-tagRFP receptor (Figure 3D). Similar results were

obtained in cells co-expressing the GHS-R1a receptor with the partially edited 5-HT_{2C}-VSV isoform (data not shown). Thus, co-expression of the 5-HT_{2C} receptor with the GHS-R1a receptor, following lentiviral transductions does not induce intracellular cAMP production and, hence, does not alter G protein coupling in Hek293 cells.

Co-localization of the 5-HT_{2C} receptor and fluorescein-ghrelin staining ex vivo

Next, endogenous co-expression of the GHS-R1a receptor and the 5-HT_{2C} receptor was investigated in rat neuronal cultures of the hypothalamus and hippocampus (Figure 4). The hypothalamus is the main brain region integrating peripheral metabolic information controlling the homeostatic regulation of appetite and food intake [70, 71]. The hippocampus is a brain structure involved in learning and memory function and has recently been linked with food intake control [72]. In addition, the 5-HT_{2C} receptor is strongly expressed in the hippocampus and on pro-opiomelanocortin (POMC) expressing neurons in the arcuate nucleus of the hypothalamus as well as in other hypothalamic regions [48, 49, 73, 74]. Moreover, a recent study by Bonn et al., demonstrates that the 5-HT_{2C} receptor can also be found on NPY producing neurons [75, 76], which was previously not recognized. In addition, a significant number of neurons in the hippocampus express the GHS-R1a receptor [51, 58, 77-79] as well as do most regions of the hypothalamus [50-52]. Specifically, in the arcuate nucleus, the GHS-R1a receptor is strongly expressed on NPY neurons, with 94% of the NPY neurons demonstrating GHS-R1a mRNA, but also on the POMC neurons , albeit only in 8% of the POMC neurons [80]. Here, we investigated the co-localization of endogenously expressed 5-HT_{2C} receptor in primary cultured neurons of rat day 17 embryos (E17), using immunocytochemistry. Serotonergic neurons develop at E16 after which mucosal enterochromaffin cells containing the largest store of mammalian serotonin start to develop [81]. Therefore, neurons were cultured from rat pups at E17 to ensure 5-HT_{2C} receptor expression. Central expression of the GHS-R1a receptor was analysed using a variation of a recently described method using fluorescein-ghrelin [82], a novel strategy to detect specific GHS-R1a receptor expression [58]. Co-localization of the 5-HT_{2C} receptor and fluorescein-ghrelin binding was correlated in primary rat hypothalamic cells (Figure 4, upper panel) as well as

primary cultures of neurons from the hippocampus (Figure 4, bottom panel). Immunostaining of the 5-HT_{2C} receptor (red) and fluorescein-ghrelin binding (green) was mainly observed in the cell bodies of both neuronal cultures. In the hypothalamus, positive cells were much less frequent but most of them co-expressed both receptors. In the hippocampus, both receptors were expressed at higher levels and cells expressing only one receptor were more frequently found. Indeed, the insert in the bottom picture shows two cells that are both positive for fluorescein-ghrelin binding to the GHS-R1a receptor but one of them lacking staining for the 5-HT_{2C} receptor (Figure 4, bottom panel). This data clearly demonstrates the co-localized endogenous expression of the GHS-R1a and 5-HT_{2C} receptor, which is a first requirement for a physical interaction between these G-protein coupled receptors *in vivo*.

Specific 5-HT_{2C} receptor blockade potentiates ghrelin's orexigenic effect in vivo

Next, we analysed the effect of specific 5-HT_{2C} receptor antagonism on ghrelin's orexigenic potential *in vivo*. Food intake of male C57Bl/6 mice was analysed following intraperitoneal administration of the specific brain-penetrant 5-HT_{2C} receptor antagonist SB242084, followed by a second intraperitoneal injection of ghrelin or vehicle (Figure 5). Repeated measures analysis revealed a significant mean effect of treatment compared to vehicle ($F_{(3,28)}$ = 6.535; p = 0.002) and a significant interaction of time × treatment (F(6.1,46.932)=3.817; p = 0.003). Post hoc analysis of the cumulative food intake indicated that the significance of ghrelin's orexigenic effect compared to vehicle tapers off after the 2 hr time point (Figure 5A). This is in line with previous findings from our lab and others demonstrating that a single administration of ghrelin causes an acute increase in food intake which is diminished over time [4, 83]. Interestingly, the significance of the ghrelin-induced increase in food intake was maintained after the 2 hr time point following SB242084-mediated 5-HT_{2C} receptor antagonism ($p<0.01$), resulting in a ghrelin-mediated increase in food intake which was still apparent at 9 hours, while the 5-HT_{2C} antagonist has no effects on food intake when administered on its own (Figure 5A). We hypothesize that the 5-HT_{2C} receptor interacts with the GHS-R1a receptor following its activation by ghrelin, potentially via a dynamic dimerization, and attenuates ghrelin's orexigenic

effect, which is in line with our *in vitro* findings (Figure2 and see [27]). Specific 5-HT_{2C} receptor antagonism maintains the significance of ghrelin's orexigenic effect following acute administration. In addition, the interaction on food intake following ghrelin and SB242084 co-administration, compared to ghrelin alone, are individually depicted in bar graphs and clearly visible at 8 and 24 hours after food placement, but not at 1hr (Figure 5B, C, D). At the 1 hr timepoint ghrelin's effect is still significant compared to control and co-administration of the 5-HT_{2C} receptor antagonist here has no additional effect on food intake. Together, these data indicate that ghrelin-induced increases in food intake can be modulated via specific 5-HT_{2C} antagonism, resulting in a longer duration of ghrelin's orexigenic effect.

Specific 5-HT_{2C} receptor agonism attenuates ghrelin's orexigenic effect in vivo

Finally, we analysed the effect of specific 5-HT_{2C} receptor agonism, using lorcaserin, on ghrelin's orexigenic effect *in vivo*. To this end, cumulative food intake of male C57Bl/6 mice following subcutaneous administration of lorcaserin with and without intraperitoneal ghrelin was analysed (Figure 6). Repeated measures analysis revealed a significant mean effect of treatment compared to vehicle ($F_{(3,29)} = 3.308$; $p = 0.034$) but no significant interaction of time × treatment ($F(5.046, 48.775)=0.956$; $p = 0.454$). Again an initial significant increase in food intake was observed following acute treatment with ghrelin compared to vehicle, which lasted up to 2 hours, after which significance tapers off (Figure 6A, B, C, D). Interestingly, ghrelin's initial orexigenic effect was not observed when animals also received the 5-HT_{2C} specific agonist, lorcaserin, at 3mg/kg. Indeed, when the 5-HT_{2C} receptor is activated using lorcaserin, the acute orexigenic effect is completely blocked in the first 2 hours. Furthermore, no effect on food intake was observed with this sub-threshold dose of lorcaserin on its own (Figure 6A). At the 8h timepoint ghrelin's orexigenic effect compared to control is no longer observed, but the combination treatment actually has a significant decreased food intake compared to ghrelin, reinforcing the significantly inhibition on ghrelin's orexigenic effect by 5-HT_{2C} receptor agonism (Figure 6D).

In summary, this study gives compelling *in vitro* and *in vivo* evidence, for a central interaction between GHS-R1a and 5-HT_{2C} receptor signalling, in line with previous findings (Schellekens et al. 2013 JCB; Hansson et al. 2014 Neuropsychopharm). It is likely that this interaction occurs in the arcuate nucleus of the hypothalamus, but whether this interaction is via dimerization on POMC or NPY neurons, where both receptors are expressed, despite GHS-R1a receptor dominance on NPY and 5-HT_{2C} receptor dominance on POMC neurons, remains to be determined. However, it is also possible that this interaction extends beyond the homeostatic hypothalamic regulation of food intake and may involve hedonic feeding behaviour. Indeed, recent studies have identified the ghrelinergic system as a key player in hedonic food intake behaviours, including the motivational drive to eat, the rewarding aspects of food intake and the stress-induced ingestion of palatable foods [84-92]. Interestingly, the 5-HT_{2C} receptor has also been implicated in reward-related behaviours [93, 94], which may explain some overlapping functionalities with the GHS-R1a receptor including involvement in the hedonic regulation of food intake. Another possibility to consider is that the interaction is not via a direct physical interaction but through an indirect mechanism mediated by the control both receptors have on the mesolimbic dopaminergic system, a key pathway for non-homeostatic feeding [95]. It has previously been shown that 5-HT_{2C} receptor agonism has an inhibitory control on dopaminergic neurons in the ventral tegmental area (VTA) through the activation of GABAergic interneurons (for review, see [96]). In addition, the GHS-R1a receptor is expressed on dopaminergic neurons in the VTA, enabling ghrelin to have a direct stimulatory effect on the mesolimbic dopaminergic system [97]. Indeed, detailed investigations into the potential interaction between GHS-R1a and 5-HT_{2C} receptor signalling through the mesolimbic pathway are now warranted.

Conclusion

Together, this study shows compelling evidence for a functionally relevant interaction between the GHS-R1a and 5-HT_{2C} receptor. Pharmacological blockade of the 5-HT_{2C} receptor enhances the duration of ghrelin-mediated increase in food intake in mice (Figure 5), which is in

line with the attenuation of ghrelin-mediated activation of the GHS-R1a *in vitro* when the 5-HT_{2C} receptor is co-expressed (Figure 2). In addition, agonism of the 5-HT_{2C} receptor, blocks ghrelin's orexigenic effect in mice (Figure 6), which may partly explain the satiety inducing effects of therapeutic doses of the 5-HT_{2C} receptor specific agonist, lorcaserin. This data uncovers a novel mechanism for fine-tuning GHS-R1a receptor-mediated food intake via serotonergic activity. These findings have important implications for the development of future pharmacological strategies in weight reduction. A more efficacious weight loss could potentially be achieved following the combined pharmacotherapeutic targeting of the ghrelinergic appetite signalling pathway and the 5-HT_{2C} receptor-mediated induction of satiety, thereby enhancing specificity and reducing side effects. Indeed, a combined pharmacological treatment to target both the GHS-R1a and 5-HT_{2C} receptor simultaneously might be a novel therapeutic approach in the treatment of eating disorders and obesity, and future investigations are warranted. In addition, a potential interaction of the GHS-R1a receptor and the 5-HT_{2C} receptor in reward centers regulating the hedonic aspects of food intake, including the VTA, is likely to broaden the application potential of novel ghrelinergic and serotonergic pharmacotherapeutics.

Methods

Receptor ligands

Ligands were prepared as previously described [27]. Briefly, the endogenous ligand of the GHS-R1a receptor, ghrelin (SP-GHRL-1; Innovagen), the non-peptide GHS-R1a receptor agonist, MK0677 (SP960334C; NeoMPS), 5–hydroxytryptamine (5-HT, H9523; Sigma), the D_1 receptor agonist, 6,7-ADTN hydrobromide (Asc-150, Ascent Scientific), and the GHS-R1a specific inverse agonist, peptide [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (SP-analog, #1946; Tocris) were prepared at a 1mM stock solution in assay buffer, consisting of 1x Hanks balanced salt solution (HBSS) supplemented with 20mM HEPES. Stock solutions were further diluted in assay buffer to the required concentration for the *in vitro* assays. MK-0677 (also known as ibutamoren, L-163,191) is a highly specific and potent full agonist of the GHS-R1a receptor, which can activate

signalling pathways at doses ranging from 0.2 – 1.4 nM) and, *in vivo,* has been shown to potently induce growth hormone (GH) and cortisol release [17, 98]. The brain penetrant 5-HT_{2C} specific antagonist SB242084 (#2901; Tocris) was prepared in DMSO as 20 mg/ml stock solution. For the *in vivo* cumulative food intake experiments stocks of ghrelin and SB242084 were further diluted in saline. The 5-HT_{2C} specific agonist, (+/-)-lorcaserin hydrochloride (FL32280; Carbosynth) was directly prepared in sterile saline.

Cell Culture

Human embryonic kidney cells (Hek293A) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 4.5 g/L glucose and L-glutamine (Sigma Aldrich, Ireland), supplemented with 10% heat inactivated foetal bovine serum (FBS). Stably transfected Hek-GHS-R1a-EGFP, Hek-5-HT_{2C}–EGFP and Hek-5-HT_{2C}–VSV-EGFP cells were cultured in complete DMEM media supplemented with 300 ng/µl G418 as maintenance antibiotic, as previously described [27]. All cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere to a confluence of >85% after which the cells were resuspended and propagated to a lower density.

Transfection and lentiviral transduction

Stably transfected Hek293A cell lines were generated following Lipofectamine LTX plus reagent (Invitrogen) mediated transfections with plasmids constructs expressing either the human GHS-R1a receptor (Accession code: U60179.1), the unedited 5-HT_{2C}-INI receptor (Genecopeia, H3309; Accession code: NM_000868) or the partly edited 5-HT_{2C}-VSV receptor isoform (Genecopeia, T0336, Accession code: AF208053.1), as previously described [27, 99]. In addition, Hek293A cells stably expressing the GHS-R1a-EGFP, the 5-HT_{2C}-EGFP or the 5-HT_{2C}-VSV-EGFP were transduced using lentiviral vectors to co-express the GHS-R1a, 5-HT_{2C}, 5-HT_{2C}-VSV or D_1 receptor constructs with a red fluorescent protein tag (RFP), as previously described [27]. Cells were transduced with the GPCR-RFP expressing lentiviral vectors diluted in transduction media, consisting of DMEM with 2% heat-inactivated FBS, 1% NEAA and an additional 8µg/ml polybrene® (Sigma; H9268). Stable expression of the EGFP fluorescently-tagged GHS-R1a

receptors was routinely monitored using flow cytometry and expression levels were not affected by co-expression of the 5-HT_{2C}-RFP construct following lentiviral transduction (data not shown). All cell lines were generated following approval and in full accordance with the Environmental Protection Agency (Ireland) under GMO register number G0331-01.

Flow cytometry fluorescence resonance energy transfer (fcFRET)

Cells were harvested 48 to 72 h after transduction using 37°C trypsin/EDTA, centrifuged and resuspended in PBS and passed through a cell strainer with 40 μm nylon mesh (BD Biosciences, #352340) prior to analysis. fcFRET analysis was performed on an LSR II cytometer (BD biosciences) and the eGFP was excited at 488 nm and detected with a 525/50 nm bandpass filter, while TagRFP was excited at 561nm and detected with a 610/20 nm bandpass filter. FRET between eGFP and TagRFP was measured by excitation at 488nm and detection with a 610/20 nm bandpass filter (i.e. excitation of the "donor" but detection of the "acceptor"). For each sample, 10^4 cells were analysed. Live cells were gated according to forward and sideward scattering (FSC/SSC). Non transduced Hek293A cells were used for background correction. Cells expressing donor or acceptor construct only were used to compensate the signal in the FRET channel for spectral bleed-through and cross-excitation. Data was analysed using FACSDiva software (BD biosciences).

Calcium mobilization assay

Receptor-mediated changes in intracellular calcium (Ca^{2+}) were analysed as previously described [27]. Briefly, stably transfected cells were seeded in black 96-well microtiter plates at a density of 2.5 x 10^5 cells/ml (2.5 x 10^4 cells/well) and maintained for ~24hrs at 37°C in a humidified atmosphere containing 5% CO_2. On the day of the assay, growth medium was aspirated off and cells were incubated with 25 μl of assay buffer (1x Hanks balanced salt solution, HBSS, supplemented with 20mM HEPES buffer) and 25 μl of Calcium 4 dye (R8141, Molecular Devices Corporation, Sunnyvale, CA) according to the manufacturer's protocol. Cells were pre-treated with 1 μM of the GHS-R1a inverse agonist, peptide [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (#1946; Tocris), contained in the assay buffer, to inhibit constitutive

GHS-R1a receptor activity. Addition of agonist (25 µl/well) was performed by the Flexstation II multiplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA), and fluorescent readings were taken for 80 seconds in flex mode with excitation wavelength of 485 nm and emission wavelength of 525 nm. The relative increase in cytosolic calcium [Ca^{2+}] was calculated as the difference between maximum and baseline fluorescence (Vmax-Vmin; the treatment-associated emission minus the unstimulated baseline emission) and depicted as percentage relative fluorescent units (RFU) compared to response as elicited by control, 3.3% fetal bovine serum (FBS). Values resulting from incorrect pipetting by the Flexstation were excluded from the analysis. Data was analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.).

Cyclic adenosine monophosphate (cAMP) assay

Intracellular 3',5'-cyclic adenosine monophosphate (cAMP) was investigated 4 days after transduction of Hek-5-HT_{2C}-INI-EGFP or Hek-5-HT_{2C}-VSV with lentiviral constructs expressing RFP tagged D_1 receptor (lvDR1-tagRFP) using the LANCE Ultra cAMP assay (PerkinElmer; #TRF0262), according to manufacturer's instructions. Briefly, 5 µl of $2*10^5$ cell/ml cell suspension was plated per well in a white 384-well plate (Perkin Elmer; Optiplate 6007291). Receptor activation was stimulated via the addition of 5 µl per well of the D_1 receptor agonists 6,7-ADTN hydrobromide (Ascent Scientific; Asc-150). Following 30 minute incubation at room temperature, 5 µl per well Eu-cAMP tracer in stimulation buffer and 5 µl per well monoclonal Ulight-anti-cAMP antibody, were added and incubated for an hour at room temperature, protected from light. Receptor mediated increases in cAMP competes with the Eu-cAMP tracer and subsequent decreases in time-resolved fluorescence resonance energy transfer (TR-FRET) emission was measured at 615 nm and 665 nm in the Synergy 2 Multi-Mode Microplate Reader (BioTek). A quench correction was performed minimizing false positives and false negatives by calculating the blank corrected ratio 665 nm/615 nm using the equation: F665,CS = [(F665,S – F 665,BL) x F615,MAX]/F615,S. The blank value is separately measured by adding buffer to the

wells to obtain blank reading at 665 nm. Data was analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.).

Embryonic primary neuronal cultures

Hypothalamic and hippocampal primary neuronal cultures were established from brains of embryonic day 17 (E17) Sprague Dawley rats generated at the animal care facility of the IMBICE. All procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA. The protocol was approved by the Institutional Animal Care and Use Committee of the IMBICE. Briefly, pregnant rats were anesthetized and prepared to aseptically remove the embryos. Each brain was removed from the skull and placed on an ice-cooled petri dish with ventral side up. A micro-dissection forceps was used to pinch out the hypothalamic region posterior to the optic chiasm, anterior to the mammillary bodies, and 3 mm deep. With the dorsal side up, a sagittal cut was made down the midline of the brain separating the left and right hemisphere. The hippocampi were pinched out from each side of the brain following removal of the brainstem and white matter. All tissue was harvested in ice-cold Hank's solution. Afterwards, cells were dissociated with a solution containing trypsin 0.25 mg/ml (cat#L2700-100, Microvet) and deoxyribonuclease I from bovine pancreas 0.28 mg/ml (cat# D5025, Sigma Aldrich) at 37 °C for 20 min, then 300 µl of FBS was added to stop the digestion and cells were mechanically dissociated using several glass pipettes with consecutive smaller tip diameters. Cells were seeded on 24 x 24 mm glasses (5 x 10^4 cells/each) previously treated with poly-L-lysine (cat# P8920, Sigma Aldrich) and laid over 6-well plates. Cells were incubated at 37 °C in a 95 % O_2 and 5% CO_2 atmosphere with DMEM/F12 1:1 medium supplemented with 10 % FBS, 0.25 % glucose, 2 mM glutamine (cat#21051-016, Gibco), 3.3 µg/ml insulin (Nordisk Pharm Ind, Inc, Clayton, North Carolina, United States), 5 U/ml penicillin G sodium salt (Richet, Buenos Aires, Argentina), 5 µg/ml streptomycin (Richet), 40 µg/ml gentamicin sulfate salt (Richet) and 1 % vitamin solution (cat#L2112-100, Microvet). On culture day 4, half of the incubation medium was replaced with medium containing cytosine β-D-arabinofuranoside (AraC) to reach a final

concentration of 5 μM (cat# C1768, SigmaAldrich). Neuronal cells were cultured for 7-10 days and then used to perform binding and immunocytochemistry assays.

Fluorescein-ghrelin binding and serotonin receptor 2C immunostaining

An *in vitro* binding assay was performed using fluorescein-ghrelin(1-18) [82] provided by Dr. Leonard Luyt from The University of Western Ontario, Canada. Specificity and accuracy of the fluorescein-ghrelin tracer as a strategy to visualize central GHS-R1a receptor expression has recently been demonstrated [58]. The 5-HT_{2C} receptor was detected with a mouse monoclonal antibody raised against the C-terminus of the receptor, previously validated for specificity in literature [100]. Briefly, neuronal culture glasses were washed once with HBSS, covered with 400 nM fluorescein-ghrelin in HBSS, incubated at room temperature for 20 min in a humidified chamber, and subsequently rinsed twice in HBSS. Cells were then fixed with 4% formaldehyde in phosphate buffered saline (PBS) pH 7.4 for 30 min at 4°C. In order to perform immunofluorescence staining, cells were treated with blocking solution (3% normal donkey serum and 0.25% TritonX in PBS) and then incubated with goat anti-fluorescein antibody conjugated to Alexa Fluor 488 (Molecular Probes, A-11096, 1:100 in blocking solution) for two days at 4°C and washed with PBS. Afterwards, cells were incubated with mouse anti-5-HT_{2C} antibody (Santa Cruz, cat# sc-17797, 1:200 in blocking solution) for 24h at 4°C, washed with PBS, and finally incubated for 1h at room temperature with donkey anti-mouse antibody conjugated to Alexa Fluor 594 (Molecular Probes, cat# A21203, 1:1000 in blocking solution) and rinsed with PBS. Negative controls were generated by omitting the primary or the secondary antibodies and no staining was found (data not shown). The slides were visualized within a week and stored at 4°C. Fluorescent and phase contrast images were acquired with a Nikon Eclipse 50i microscope and a DS-Ri1 Nikon digital camera. The open-source image editing software FIJI was used to adjust contrast and brightness of microphotographs and to prepare the composite panels [101].

Cumulative food intake

Male C57Bl/6 mice (Harlan, UK) were housed per four in standard holding cages at the animal care facility of University College Cork. The holding room temperature (21±1 °C) and humidity (55±10%) were controlled under a 12-h light/dark cycle (lights on 7.00 AM, lights off 7.00 PM). Water and food (2018S Teklad Global 18% Protein Rodent Diet) were available *ad libitum* throughout the study. The mice were habituated on three independent days to the experimental settings. Cumulative food intake studies were performed based on protocols described in previous studies (Asakawa, Inui et al. 2001; Finger, Schellekens et al. 2011). Briefly, the mice were weighed, single-housed in new cages in the experimental room and habituates for 20 minutes before injections. To investigate the effect of 5-HT_{2C} receptor antagonism on ghrelin's orexigenic effect a cohort of 32 mice, n=8 per group, of approximately 11 week old animals were used. For the first injection, SB242084 (#2901; Tocris) (2.0 mg/kg in saline and 1.0% DMSO) and vehicle (saline with 1.0% DMSO) and for the second injection ghrelin (SP-GHRL-1; Innovagen) (200 nmol/kg in saline) and vehicle (saline) were administered via intraperitoneal (IP) administration (10 µl/gram of body weight). To investigate the effect of 5-HT_{2C} receptor agonism on ghrelin's orexigenic effect a cohort of 35 mice, n=7-10 per group, of approximately 10 week old mice were used. First, the dose response effect (0, 1, 3 and 10 mg/kg) of a racemic mixture of the 5-HT_{2C} receptor agonist, (+/-)-lorcaserin hydrochloride (FL32280; Carbosynth) on cumulative food intake was established following a 16 hr food restriction (data not shown). The sub-threshold dose of 3 mg/kg (0.3 mg/ml) was selected for further experiments as no effect on food intake was observed using this dose for up to 8 hours. For combination experiment, (+/-)-lorcaserin hydrochloride (FL32280; Carbosynth) (3.0 mg/kg in saline) and vehicle (saline) were administered subcutaneously (10 µl/gram of body weight) followed by a second injection ghrelin (Innovagen; SP-GHRL-1) (200 nmol/kg in saline) and vehicle (saline) via intraperitoneal (IP) administration (10 µl/gram of body weight). Time between the first and second injection was 15 minutes and pre-weighed chow food pellets were carefully placed in the experimental cages 20 minutes following the second IP injection. Thereafter, the amount of food was weighed at regular time intervals (20 min, 40 min, 1 h, 1h30min, 2 h, 3 h, 4 h, 5 h, 6 h, 7

h, 8 h, 9 h and 24 h). Animals that crumbled the pellet or wetted the pellet, which were both rare occasions, were excluded to ensure differences in weight reflect pellet consumed. At the end of the experiment the mice were placed back in their original cages in the holding room. Cumulative food intake was analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.). All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS statistics 20, Chicago, IL, U.S.A.). For *in vitro* assays, significance was determined a two-way ANOVA at a significance level of $p < 0.05$. For food intake experiments, significant difference was determined with a general linear model repeated measurement combined with a one-way ANOVA with LSD post hoc test for each timepoint. If the data was non-spherical a Huynh-Feldt correction was applied. Graphs were expressed as mean ± SEM. Statistical significances were depicted as follows: * indicating $p<0.05$, ** indicating $p<0.01$ or *** indicating $p<0.001$.

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FIGURE LEGENDS

Figure 1 FRET between the 5-HT$_{2C}$ and GHS-R1a receptor. Hek293A cells stably expressing the 5-HT$_{2C}$ receptor as an eGFP fusion protein or the partially edited 5-HT$_{2C}$ isoform, 5-HT$_{2C}$-VSV-eGFP, were transiently transduced with lentiviral vectors expressing control-TagRFP or GHS-R1a-TagRFP. Cells were analysed 72 hrs post transduction using LSRii flow cytometry. Dot plots are representative of three independent experiments. Percentages indicate levels of TagRFP expression (TagRFP vs eGFP plots) or FRET levels as a percentage of TagRFP expression (FRET vs eGFP plots).

Figure 2 Co-expression of the 5-HT$_{2C}$ receptor attenuates GHS-R1a-mediated intracellular calcium mobilization. The ligand-mediated intracellular calcium increase in Hek293A cells stably expressing the GHS-R1a receptor only (solid bars) was reduced when co-expressing the 5-HT$_{2C}$ receptor (striated bars), following exposure to different concentrations of ghrelin (A) or different concentrations of the synthetic agonist, MK0677 (B). Intracellular calcium mobilization was depicted in relative fluorescence units (RFU) as a percentage of maximal calcium increase as elicited by the control (3.3 % FBS). Graph represents the mean ± SEM of triplicate samples. Statistical significance of ligand-mediated calcium mobilization obtained in double expressing cells compared to cells solely expressing the GHS-R1a receptor is denoted as * indicating $p<0.05$, ** indicating $p<0.01$ or *** indicating $p<0.001$.

Figure 3 Co-expression of the 5-HT$_{2C}$ receptor and the GHS-R1a receptor does not influence cAMP signalling. The dopamine D$_1$ receptor agonist, 6,7-ADTN hydrobromide (0.5 nM), induces an increase in cAMP in human embryonic cells transiently expressing the D$_1$ receptor following lentiviral transduction (lvDRD1-tagRFP) but not in cells stably expressing the 5-HT$_{2C}$ receptor (A) or the GHS-R1a receptor (B). Co-expression of the GHS-R1a receptor, following lentiviral transduction (lvGHS-R1a-EGFP) in cells stably expressing the 5-HT$_{2C}$

receptor does not induce intracellular cAMP production (C). Neither does lentiviral co-expression of 5-HT_{2C} receptor (lv5HT_{2C}-EGFP) in cells stably expressing the GHS-R1a receptor (D). Intracellular basal (nonstimulated) cAMP level was used for comparison (black bars). The data is depicted as the mean ± SEM with each concentration point performed in triplicate. Statistical significance is denoted as *a*= p<0.001 compared to vehicle (-) and *b*= p<0.001 compared to 5-HT (A) or ghrelin (Ghrl) (B), respectively.

Figure 4 Co-localization of the 5-HT_{2C} receptor and ghrelin-fluorescein staining in rat hippocampal and hypothalamic neurons. Primary cultured hypothalamic (top panel) and hippocampal (bottom panel) cells were shown to express the 5-HT_{2C} receptor, indicated in red, and to also bind fluorescein-ghrelin, indicated in green. Overlapping expression is indicated in yellow. Nuclear stain by bisbenzimide is indicated in blue. Data is representative of three independent staining experiments of primary cultured hippocampal neurons (left and right) from day 17 rat embryos (E17).

Figure 5 Specific 5-HT_{2C} receptor antagonism potentiates ghrelin's orexigenic effect *in vivo.* Cumulative food intake (A) and food intake at time-points 1, 8 and 24 hour (B, C, D) are depicted for *ad libitum* fed male C57Bl/6 mice following intraperitoneal administration of the brain-penetrant 5-HT_{2C} receptor antagonist SB242084 (2 mg/kg) or vehicle 1 (saline+ 1% DMSO) followed by ghrelin (200 nmol/kg) or vehicle 2 (saline). Results are depicted ± SEM. Statistical significant differences compared to Vehicle-Vehicle (A) and between all groups (B, C, D) at each time point are depicted as * indicating $p<0.05$, ** indicating $p<0.01$ or *** indicating $p<0.001$, n=8 per group.

Figure 6 Specific 5-HT_{2C} receptor agonism attenuates ghrelin's orexigenic effect *in vivo.* Cumulative food intake (A) and food intake at time-points 20 min, 1 and 8 hour (B, C, D) are depicted for *ad libitum* fed male C57Bl/6 mice following subcutaneous

administration of the 5-HT_{2C} specific agonist lorcaserin (3 mg/kg) or vehicle 1 (saline; 1% DMSO) followed by intraperitoneal ghrelin (200 nmol/kg) or vehicle 2 (saline). Results are depicted ± SEM. Statistical significant differences compared to Vehicle-Vehicle (A) and between all groups (B, C, D) at each time point are depicted as * indicating $p<0.05$, ** indicating $p<0.01$ or *** indicating $p<0.001$, n=7-10 per group.

851 FIGURES

852

853 FIGURE 1

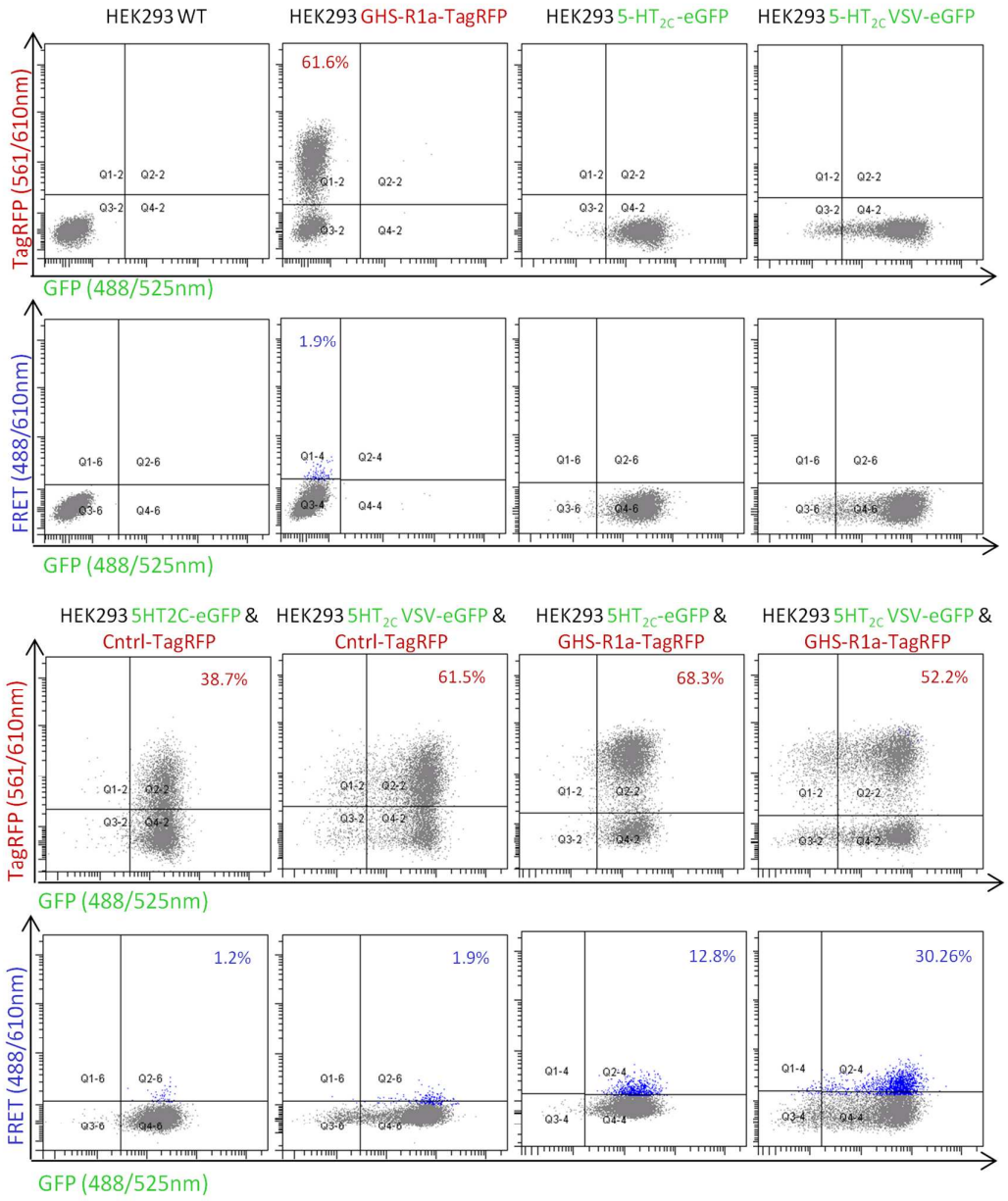

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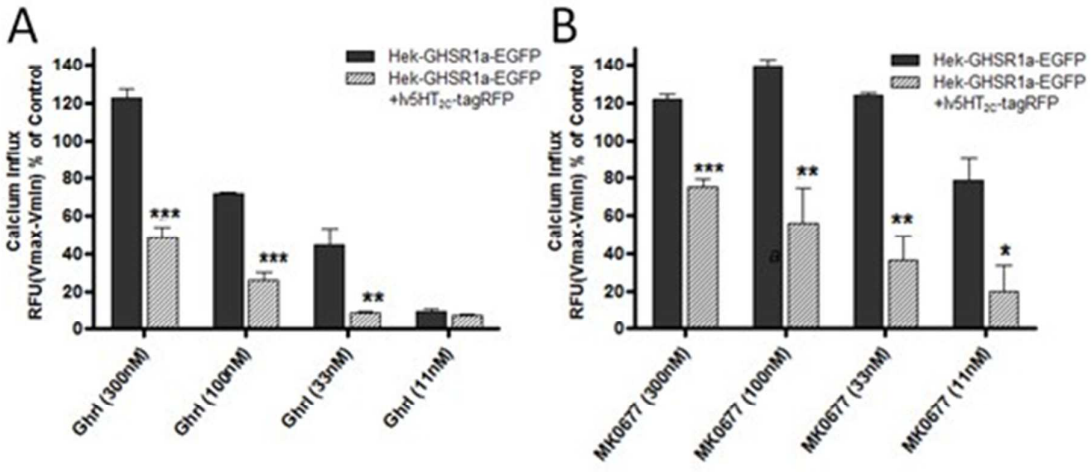
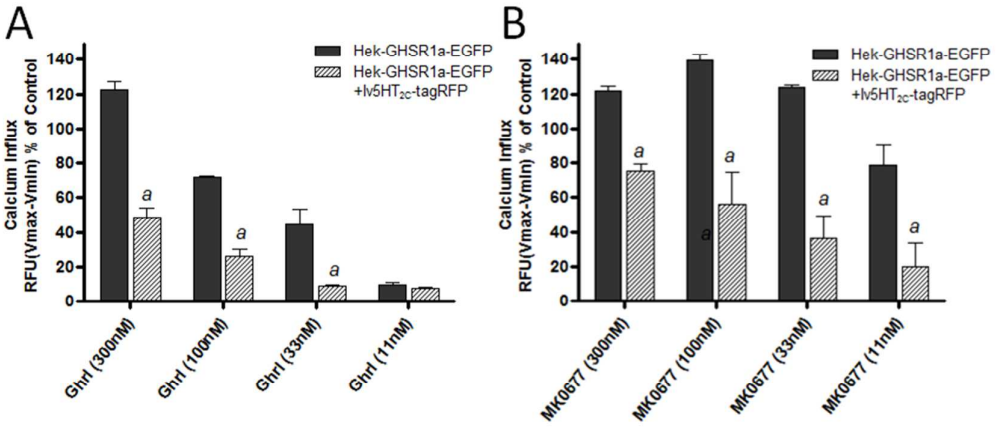
FIGURE 2


A
B
Hek-GHSR1a-EGFP
Hek-GHSR1a-EGFP +hr5HT2C-tagRFP
Calcium Influx RFU(Vmax-Vmin) % of Control
Ghrl (300nM)
Ghrl (100nM)
Ghrl (33nM)
Ghrl (11nM)
MK0677 (300nM)
MK0677 (100nM)
MK0677 (33nM)
MK0677 (11nM)


A
B
Hek-GHSR1a-EGFP
Hek-GHSR1a-EGFP +hr5HT2C-tagRFP
Calcium Influx RFU(Vmax-Vmin) % of Control
Ghrl (300nM)
Ghrl (100nM)
Ghrl (33nM)
Ghrl (11nM)
MK0677 (300nM)
MK0677 (100nM)
MK0677 (33nM)
MK0677 (11nM)

858 FIGURE 3

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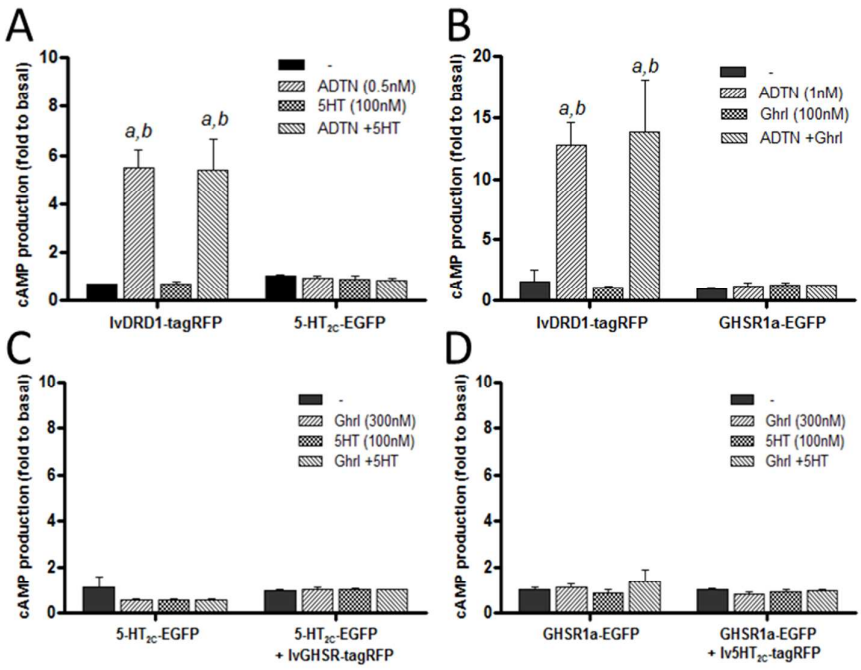

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861 FIGURE 4

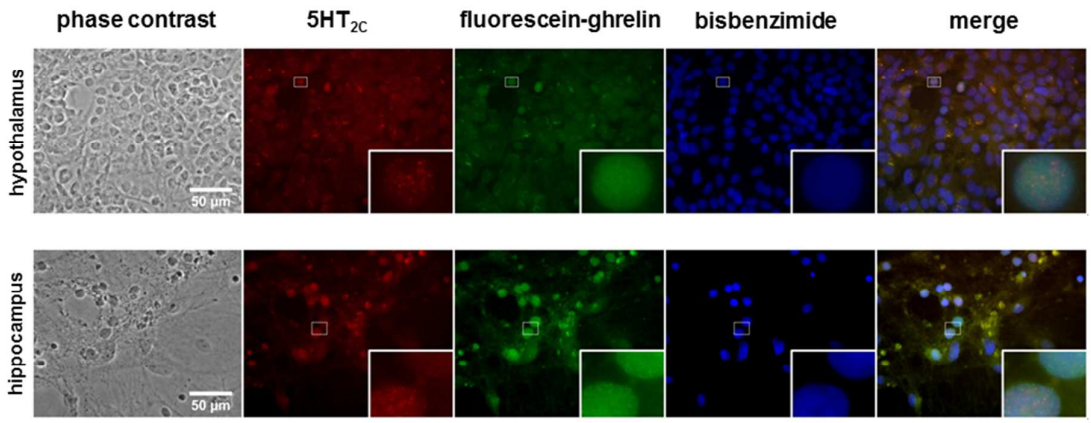

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FIGURE 5

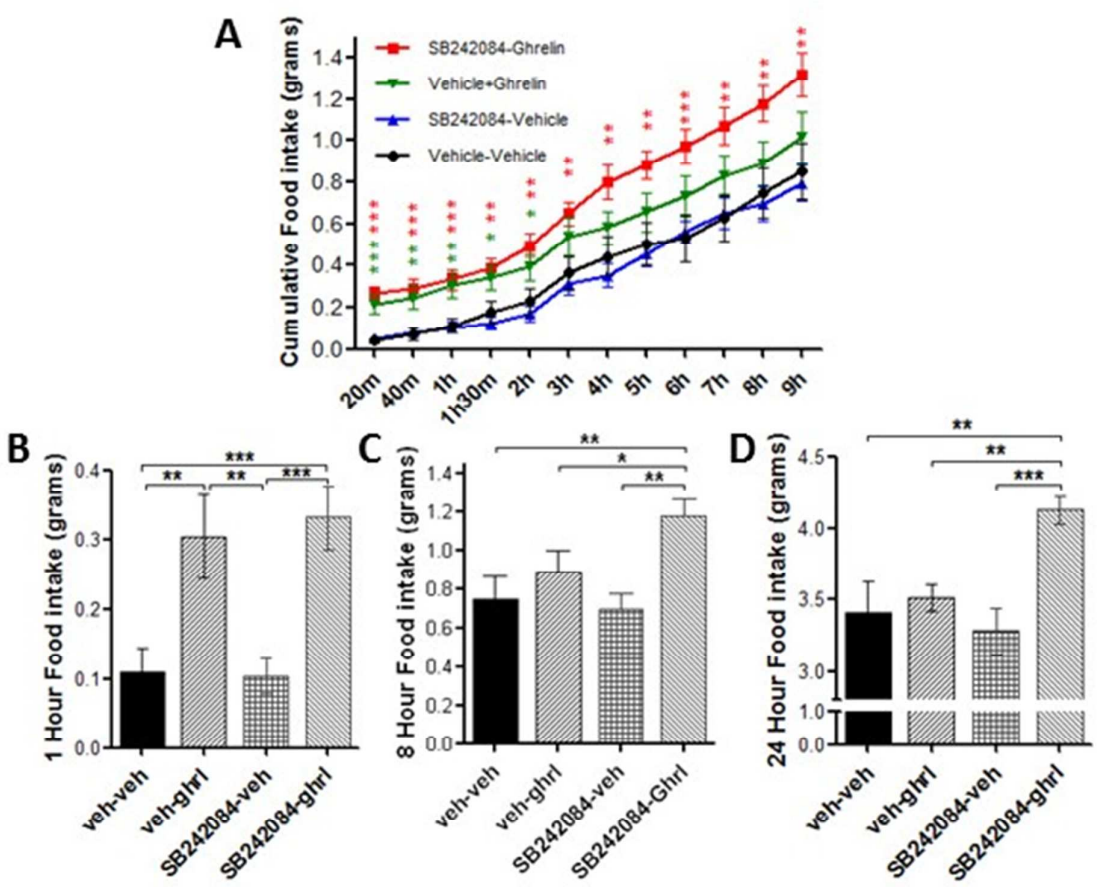
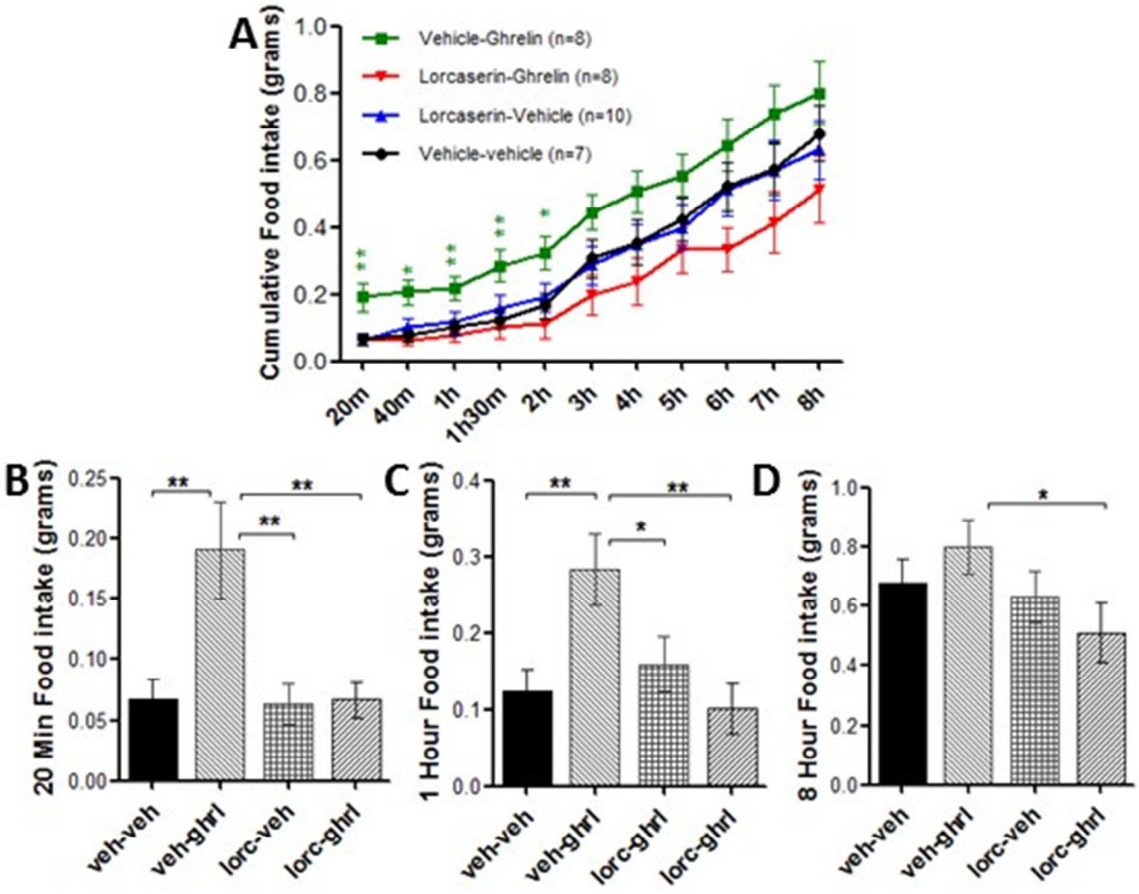

A
SB242084-Ghrelin
Vehicle+Ghrelin
SB242084-Vehicle
Vehicle-Vehicle
Cumulative Food intake (grams)
20m
40m
1h
1h30m
2h
3h
4h
5h
6h
7h
8h
9h
B
1 Hour Food intake (grams)
C
8 Hour Food intake (grams)
D
24 Hour Food intake (grams)
veh-veh
veh-ghrl
SB242084-veh
SB242084-Ghrl

FIGURE 6


A
Cumulative Food intake (grams)
Vehicle-Ghrelin (n=8)
Lorcaserin-Ghrelin (n=8)
Lorcaserin-Vehicle (n=10)
Vehicle-vehicle (n=7)
20m
40m
1h
1h30m
2h
3h
4h
5h
6h
7h
8h
B
20 Min Food intake (grams)
C
1 Hour Food intake (grams)
D
8 Hour Food intake (grams)
veh-veh
veh-ghrl
lorc-veh
lorc-ghrl

871 TABLE OF CONTENTS GRAPHIC

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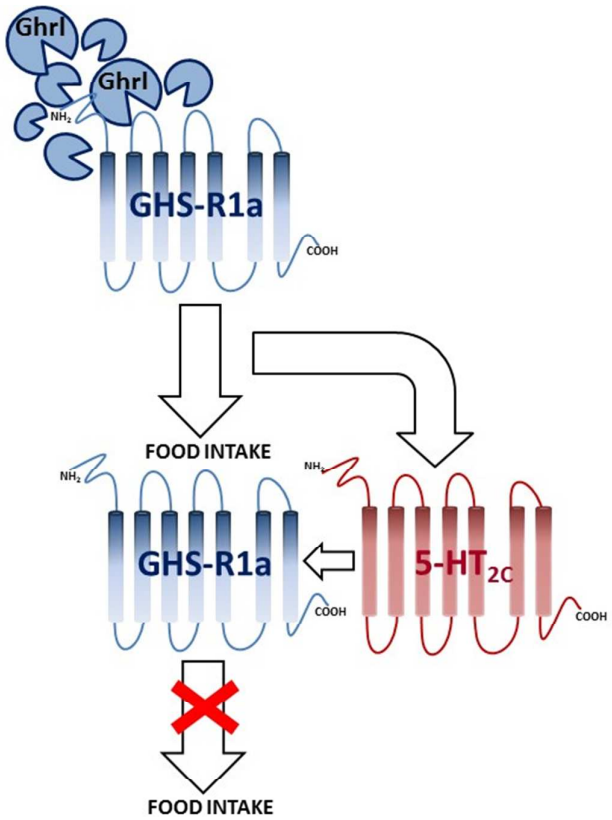

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