Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signalling *

Harriët Schellekens1,5, Wesley E.P.A. van Oeffelen5, Timothy G. Dinan1,2,3, and John F. Cryan1,2,4.

1Food for Health Ireland, 2Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, 3Dept of Psychiatry, 4Dept of Anatomy and Neuroscience, 5School of Pharmacy, University College Cork, Cork, Ireland

*Running Title: A novel heterodimer of the GHS-R1a and the 5-HT2C receptor

To whom correspondence should be addressed: Professor John F. Cryan, Department of Anatomy and Neuroscience, Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, Food for Health Ireland, University College Cork, College Rd., Cork, Ireland, Tel +353 21 490 5426; Fax +353 21 427 3518; E-mail: j.cryan@ucc.ie

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Background: The ghrelin receptor (GHS-R1a) has multiple biological functionalities, including the regulation of appetite and hedonic food intake.

Results: A novel GHS-R1a/5-HT2C heterodimer was identified, in addition to GHS-R1a/D1 and GHS-R1a/MC3. In addition, a novel heterodimer between the GHS-R1a receptor with the D1 (dopamine 1) receptor as well as that of the GHS-R1a-MC3 heterodimer. In addition, a novel heterodimer between the GHS-R1a receptor and the 5-HT2C receptor was identified. Interestingly, dimerization of the GHS-R1a receptor with the unedited 5-HT2C-INI receptor, but not with the partially edited 5-HT2C-VSV isoform, significantly reduced GHS-R1a agonist mediated calcium influx, which was completely restored following pharmacological blockade of the 5-HT2C receptor. These results combined, suggest a potential novel mechanism for fine-tuning GHS-R1a receptor mediated activity via promiscuous dimerization of the GHS-R1a receptor with other GPCRs involved in appetite regulation and food reward. These findings may uncover novel mechanisms of significant relevance for the future pharmacological targeting of the GHS-R1a receptor in the homeostatic regulation of energy balance and in hedonic appetite signalling, which both play a significant role in the development of obesity.

SUMMARY

G-protein coupled receptors (GPCRs), such as the ghrelin receptor (GHS-R1a), the melanocortin 3 receptor (MC3) and the serotonin 2C receptor (5-HT2C), are well-known for their key role in the homeostatic control of food intake and energy balance. Ghrelin is the only known gut-peptide exerting an orexigenic effect and has thus received much attention as an anti-obesity drug target. In addition, recent data has revealed a critical role for ghrelin in dopaminergic mesolimbic circuits involved in food reward signalling. This study investigates the downstream signalling consequences and ligand-mediated co-

The growth hormone secretagogue (GHS-R1a) receptor was initially described as an orphan receptor, activated by synthetic peptidyl growth hormone secretagogues (GHS), such as herexalin, growth hormone
releasing peptides (GHRP1, GHRP2 and GHRP6) and the nonpeptidyl ligand, MK-0677, which were all shown to stimulate the release of growth hormone (GH) from the pituitary [1-3]. Shortly thereafter, the gastric-derived-peptide ghrelin was identified as the endogenous ligand for the GHS-R1a receptor by a reverse pharmacological approach [4], subsequently designating the GHS-R1a receptor as the ghrelin receptor [5]. The GHS-R1a receptor is expressed in both the periphery and central nervous system and, when activated by ghrelin, it mediates a multitude of biological activities, including the secretion of GH as well as the stimulation of appetite and food intake, maintaining the body’s energy homeostasis (for review see [6]). Due to its orexigenic effect, the ghrelinergic system has received attention as a promising anti-obesity therapeutic target [6-14]. In addition, recent studies identified a pivotal role for the ghrelinergic system in additional food intake behaviours, including reward signalling following ingestion of palatable food as well as the motivational drive to eat (for review see [15-20]). Expression of the GHS-R1a receptor in the extra-hypothalamic neurocircuitry, regulating this non-homeostatic feeding, including the ventral tegmental area (VTA), nucleus accumbens (NAcc), hippocampus and amygdala, is in line with ghrelin’s role in the hedonic aspects of food intake [21, 22].

The GHS-R1a receptor is a G-protein coupled receptor (GPCR) belonging to class I of GPCRs [23, 24]. GPCRs, such as the GHS-R1a, have been found to crosstalk with other GPCRs and have been found to exist and function as dimers or even higher-structure oligomeric complexes [25-28]. Heterodimerization of the GHS-R1a receptor with other GPCRs involved in the homeostatic or hedonic regulation of food intake may be able to explain crosstalk between neuropeptide systems and could potentially serve to modulate specific GHS-R1a mediated signalling pathways and functionalities. Recent studies support these notions by demonstrating the existence of heterodimers of the GHS-R1a receptor with the melanocortin 3 receptor (MC₃) [29, 30], which is an important downstream signalling receptor in the homeostatic control of food intake and energy balance [31-34]. In addition, the involvement of a dimer between the GHS-R1a and the dopamine D2 receptor in the regulation of appetite has recently been shown [35]. Moreover, accumulating evidence supports dimerization of the dopamine D₂ receptor with the GHS-R1a receptor, leading to enhanced dopamine signalling [36]. The rewarding and pleasurable aspects of palatable food are primarily mediated via neuronal dopamine release in the mesolimbic circuitry system [37]. Interestingly, ghrelin has also been shown to enhance food reward [15, 20, 38] and this effect may well be mediated via dimerization with dopamine receptors.

The serotonin 2C (5-HT₂C) receptor is another centrally expressed GPCR involved in satiety signalling [11-14, 39-42]. Interestingly, interactions between the serotonin and ghrelin signalling pathways have been previously described. For example, pharmacological increases of brain serotonin levels and 5-HT₂C receptor agonism were shown to inhibit the increase in plasma active ghrelin in response to an overnight fast in mice, suggesting the existence of a negative feedback mechanism [43]. In addition, ghrelin has been shown to inhibit serotonin release in rat hypothalamic synaptosomes [44]. Moreover, the 5-HT₂C receptor has been identified in the regulation of reward-related behaviours [45, 46], demonstrating overlapping functionalities with the GHS-R1a receptor in the homeostatic as well as the hedonic regulation of food intake. In addition, the neuronal 5-HT₂C Receptor [47, 48] has an overlapping expression profile to the neuronal circuits expressing the GHS-R1a receptor [21, 22, 49]. A recent study demonstrated that an increase in serotonin (5-hydroxytryptophane, 5-HT) via direct administration, effectively blocked ghrelin’s orexigenic actions in rats [50]. In the same study, a similar attenuation of ghrelin induced food intake was observed following administration of the 5-HT₂ receptor agonist 5-dimethoxy-4-idoamphetamine (DOI). This serotonin-mediated attenuation of ghrelin signalling may potentially involve dimerization of the GHS-R1a and the 5-HT₂C receptor, which is a previously unexplored possibility under investigation here. Notably, serotonin receptors have been found to exists
as oligomer complexes with other serotonin receptor family members [51], with metabotropic glutamate receptors [52-54] and with dopamine receptors [55]. However, oligomeric complex formation does not always translate into second messenger signalling effects, as downstream consequences were unaffected in a 5-HT<sub>2A</sub>/mGlu<sub>2</sub> heterocomplex [54].

Further elucidating the promiscuous heterodimerization of the GHS-R1a receptor with candidate GPCRs might lead to a better understanding of the fine-tuning of GHS-R1a receptor activity as well as crosstalk within neuronal circuits, which may ultimately lead to new therapeutic intervention strategies to reduce food intake. Therefore, the current study aims to investigate the potential of the GHS-R1a receptor to dimerize with the 5-HT<sub>2C</sub> receptor through analysis of colocalized expression in vitro in heterologous cells. Receptor colocalization is the primary requirement for the existence of potential receptor dimers, as without colocalized expression there would be no possibility for dimerization in normal physiology. In addition, GHS-R1a mediated downstream calcium signalling and ligand-mediated receptor trafficking following co-expression of the GHS-R1a receptor with the D<sub>1</sub> and the 5-HT<sub>2C</sub> receptor will be analysed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, transfection and lentiviral transduction**—Human embryonic kidney cells (Hek293A) were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% non-essential amino acids (NEAA) in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. Cells were maintained to a confluence of >85% after which the cells were passaged to a lower density. Hek293A cells were transfected with a plasmid construct expressing the human GHS-R1a receptor (Genecopeia, X0963; Accession code, U60179.1), the unedited 5-HT<sub>2C</sub>-INI receptor (Genecopeia, H3309; Accession code, NM_000868) or the partly edited 5-HT<sub>2C</sub>-VSV receptor isoform (Genecopeia, T0336, Accession code: AF208053.1), each including a C-terminal-EGFP tag, using lipofectamine LTX plus reagent (Invitrogen), according to manufacturer’s instructions. Constructs contained the neomycin resistance marker and cells stably expressing the GPCR-EGFP fusion proteins were selected using geneticin (G418, Merck) as a selection antibiotic. Cells with the highest CMV promoter-mediated expression of the insert gene tagged with the C-terminal EGFP were further selected using flow assisted cell sorting (FACS) after which they were maintained in complete DMEM media, supplemented 300ng/ul G418 as maintenance antibiotic. In addition, HEK293A cells stably expressing the GHS-R1a-EGFP, the 5-HT<sub>2C</sub>-EGFP or the 5-HT<sub>2C</sub>-VSV-EGFP were transduced using in-house generated lentiviral vectors to co-express the GHS-R1a, 5-HT<sub>2C</sub>, 5-HT<sub>2C</sub>-VSV, D<sub>1</sub> or MC<sub>3</sub> constructs with a red fluorescent protein tag (RFP). Transduction was performed using a 3rd generation packaging, gene delivery and viral vector production system developed by Naldini and colleagues [56-60]. All GPCR gene constructs were cloned into a HIV-based, replication deficient, lentiviral expression plasmid pHRSIN-BX-RFP. This vector was generated from a pHRSIN-BX-IRES-EmGFP vector (kind gift of Adrian Thrasher, Institute of Child Health, London, United Kingdom), which was modified in our lab to exclude the shRNA U6 promoter and contain a RFP amplified from the pTagRFP-N vector (FP142, Evrogen) instead of an IRES-EmGFP. HIV-based lentivector (LV) particles, pseudotyped with the vesicular stomatitis virus G (VSV-G), expressing the G-protein coupled receptor (GPCR) constructs from a spleen focus-forming virus (SFFV) promoter in conjunction with the RFP sequence were produced using 293T-17 cells, following transient cotransfection of the cloned expression constructs, pHRGPCR-RFP, the packaging construct, pCMV△R8.91 and the envelope construct, pMD,G-VSVG. 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).
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Fluorescence was monitored using flow cytometry as indicator of receptor expression.

Receptor ligands—Endogenous agonists ghrelin (ghrl, 1465; Tocris), MK0677 (SP960334C; NeoMPS), 5-hydroxytryptamine (SHT, H9523; Sigma), 6,7-ADTN hydrobromide (Asc-150, Ascent Scientific), [Nle\textsubscript{4},D-Phe\textsubscript{7}]-\textalpha-MSH (3013, Tocris) and the peptide [D-Arg\textsubscript{1}, D-Phe\textsubscript{5},D-Trp\textsubscript{7,9}, Leu\textsubscript{11}]-substance P (SP-analog, 1946; Tocris) were prepared in assay buffer (1x Hanks balanced salt solution, HBSS, supplemented with 20mM HEPES). The 5-HT\textsubscript{2C} specific antagonist RS10222 (1050, Tocris) was prepared in DMSO as 10mM stock solutions before diluting into assay buffer.

Colocalization and internalization of GHS-R1a, D\textsubscript{1}, MC\textsubscript{3} and 5-HT\textsubscript{2C} receptors—Transgenic Hek293A cells stably overexpressing the GHS-R1\textalpha-EGFP receptor were transduced with lentiviral vectors lvDR1-tagRFP, lvMC\textsubscript{3}-tagRFP, lv5-HT\textsubscript{2C}-INI-tagRFP or lv5-HT\textsubscript{2C}-VSV-tagRFP to assess colocalized expression. In addition, transgenic Hek293A cells stably overexpressing the 5-HT\textsubscript{2C}-INI-EGFP or the edited isoform 5-HT\textsubscript{2C}-VSV-EGFP were transduced with lvGHS-R1\textalpha-tagRFP. The cells were transduced with viral vectors as described and seeded at 5\texttimes10\textsuperscript{4} cells/well in a total volume of 70uL to 100uL on poly-L lysine (P4707, Sigma) coated borosilicate glass slides (631-0150, VWR International) in 24-well plates. After 3 hours incubation at 37°C, 5.0% CO\textsubscript{2}, wells were flooded with DMEM, containing 10% FBS, 1% NEAA and penicillin/streptomycin, and incubated further. Colocalization was assessed using laser scanning confocal fluorescent microscopy (FV 1000 Confocal System, Olympus). To determine if GPCRs also co-internalize, receptors were ligand activated and cell fluorescent translocation was monitored on an inverted microscope (CKX41; Olympus) set-up with a sensitive XM10 camera (C-BUN-F-XM10-BUNDLE) with an infrared (IR) cut filter, mercury burner (USH-103OL) and fluorescence condenser (CKX-RFA; Olympus) converting the microscope to a 1.4 megapixel cooled monochrome CCD digital microscope. Both the green and red fluorescence of the cells were captured and merged for analysis using Cell\textsuperscript{F} software. Ligand-mediated co-internalization of the receptor pairs was quantified using the Image J software (1.45s). In each merged image 5 individual cells co-expressing the GHS-R1a and the partner GPCR were selected and fluorescence intensity of plasma membrane versus perinuclear receptor expression was determined. The single highest intracellular pixel was compared to membrane pixel intensity along a straight line axis in each selected cell. The average pixel intensity ratio of each treatment was expressed as the mean ± SEM.

Calcium mobilization assay—Receptor mediated changes in intracellular calcium (Ca\textsuperscript{2+}) were monitored on a Flex station I\textsuperscript{I}I multiplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA). Stably transfected cells were seeded in black 96-well microtiter plates at a density of 2.5 \times 10\textsuperscript{5} cells/ml (2.5 \times 10\textsuperscript{4} cells/well) and maintained for ~24hrs at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}. After removal of the growth medium, cells were incubated with 25 uL of assay buffer (1x Hanks balanced salt solution, HBSS, supplemented with 20mM HEPES buffer) and 25 uL of Ca4 dye (R8141, Molecular Devices Corporation, Sunnyvale, CA) according to the manufacturer’s protocol. Addition of ligand, prepared in assay buffer (25uL/well) was performed by the Flexstation II and fluorescent readings were taken for 160 seconds in flex mode with excitation wavelength of 485 nm and emission wavelength of 525nm. The relative increase in cytosolic calcium [Ca2+] was calculated as the difference between maximum and baseline fluorescence (V\textsuperscript{max}-V\textsuperscript{min}; the treatment-associated emission subtracted with the unstimulated baseline emission), corrected for background fluorescence, and depicted as percentage relative fluorescent units (RFU) compared to maximum response (100%). Each agonist dose response curve was constructed using GraphPad Prism software (PRISM 4.0; GraphPAD Software Inc.) using nonlinear regression analysis with variable slope. Values resulting from obvious incorrect pipetting by the flexstation were excluded from the analysis.
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**Statistical analysis** — Statistical differences were analyzed using one-way or two-way analysis of variance (ANOVA) where appropriate. Statistical significant differences were subsequently depicted as follows: * indicating p<0.05; ** indicating p<0.01 and *** indicating p<0.001, or as indicated in figure legends.

**RESULTS**

**Colocalization of the GHS-R1a receptor with candidate GPCRs**—Human embryonic kidney (HEK293A) cells, generated to stably express a functional human GHS-R1a receptor as an EGFP fusion protein, were transduced with lentiviral vectors to co-express several other GPCRs involved in appetite and satiety regulation, expressed as RFP fusion proteins. Consequently, all cells expressed the GHS-R1a receptor, while most, but not all cells expressed the candidate GPCR. Successful lentiviral transduction and RFP-tagged receptor expression was confirmed using flow cytometry (data not shown). Using fluorescent laser scanning confocal microscopy, colocalization could be demonstrated between the GHS-R1a receptor and the D\textsubscript{1} receptor (Figure 1A), the GHS-R1a and the MC\textsubscript{3} receptor (Figure 1B) as well as between the GHS-R1a and two distinct isoforms of the 5-HT\textsubscript{2C} receptor, the unedited variant 5-HT\textsubscript{2C}-INI (Figure 1C) and a partially edited isoform 5-HT\textsubscript{2C}-VSV (Figure 1D). Colocalization of the GHS-R1a with the MC\textsubscript{3} receptor was shown to be mostly intracellular while GHS-R1a and D\textsubscript{1} colocalization was ubiquitous. Co-localization of other receptor pairs following in vitro overexpression (e.g. the 5-HT\textsubscript{2C} receptor with the MC\textsubscript{3} receptor), was also investigated but failed to show overlapping expression (data not shown).

**Co-internalization of GHS-R1a heterodimers**— The GHS-R1a receptor demonstrated to colocalize with the D\textsubscript{1} receptor (Figure 2), the MC\textsubscript{3} receptor (Figure 3) and the 5-HT\textsubscript{2C} receptor (Figure 4). In addition, receptor co-internalization was shown following agonist treatment for all receptor pairs. Agonist-mediated receptor trafficking was subsequently quantified for all receptor pairs and statistical significant co-internalization was in line with the visual observations (Figure 2P, 3P and 4P).

Clear co-internalization of the GHS-R1a/D\textsubscript{1} receptor pair was observed following treatment with the D\textsubscript{1} agonist 6,7-ADTN hydrobromide (Figure 2G), which was shown to be significant (p<0.05) compared to control and the GHS-R1a receptor-specific inverse agonist, [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (SP-analog) (Figure 2P). Exposure to the SP-analog alone had no effect on receptor distribution compared to the control (Figure 2P). Moreover, the endogenous GHS-R1a agonist ghrelin and the synthetic agonist MK0677 demonstrated not only internalization of the GHS-R1a receptor (Figure 2H and I), but the D\textsubscript{1} receptor was co-internalized into the endosomes (Figure 2C and D). Overlay images confirmed colocalized expression of both receptors (Figure 2K to O). Co-internalization of the GHS-R1a/D\textsubscript{1} receptor pair was significant in response to both ghrelin (p<0.001) and MK0677 (p<0.01), respectively (Figure 2P). This reinforces the concept of heterodimerization of the GHS-R1a receptor with the D\textsubscript{1} receptor, as the individual receptors are normally not responsive towards agonists activating the partnering GPCR.

Next, co-internalization was observed for the GHS-R1a/MC\textsubscript{3} pair (Figure 3), which was shown to be significant compared to control following treatment with both ghrelin (p<0.01) and MK0677 (p<0.01) but not upon treatment with the MC\textsubscript{3} agonist, [Nle4,D-Phe7]-\alpha-MSH (Figure 3P). Noteworthy, however, was the high basal cytosolic localization of the GHS-R1a/MC\textsubscript{3} receptor pair (Figure 3A,F and K). This demonstrates an altered cellular localization upon co-expression, considering the GHS-R1a receptor is expressed on the cell membrane when expressed on its own (data not shown) and under basal conditions upon co-expression with other GPCRs (Figure 2F and 4F). Colocalization of both receptors in the cytosol was observed after merging of the obtained images (Figure 3K to O). The strong cytosolic presence of both receptors suggests heterodimerization of the GHS-R1a receptor with the MC\textsubscript{3} receptor. Treatment with the SP-analog (Figure 3E,J and O), although not significant, slightly reduced the high intracellular expression, leading to a
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significant different colocalization of the GHS-R1a/MC$_3$ pair following exposure to [Nle4,D-Phe7]-α-MSH (p<0.01), when comparing to SP-analog (Figure 3P). Finally, colocalization and co-internalization of the GHS-R1a/5-HT$_{2C}$ receptor pair was investigated (Figure 4). Treatment with 5-HT (Figure 4B,G and L), with ghrelin (Figure 4C,H and M) or with MK0677 (Figure 4D,I and N) caused an increase of membrane bound GHS-R1a and 5-HT$_{2C}$ receptor (Figure 4E,J and O). However, statistical analysis revealed that only co-internalization following ghrelin exposure was significant (p<0.05) (Figure 4P). Treatment with MK0677 and 5HT, although showing an elevated internalized intensity, did not reach statistical significance (Figure 4P). However, these cells also had elevated internalized expression under control conditions (compare Figure 2P with 4P), explaining the absence of significance. When comparing the MK0677-mediated receptor co-internalization to cells exposed to SP-analog, statistical significance was reached (p<0.05) and significance for ghrelin-mediated co-internalization was potentiated (p<0.001). This data supports the existence of a novel heterodimer between the GHS-R1a and the 5-HT$_{2C}$ receptor.

Functional interaction of the GHS-R1a receptor with the D$_1$ and MC$_3$ receptor—Both the MC$_3$ and the D$_1$ receptors are coupled to the G protein G$_{as}$, and receptor ligand binding subsequently activates adenylyl cyclase, leading to increasing intracellular concentrations of the second messenger cyclic adenosine monophosphate (cAMP). Cell lines transduced with either the D$_1$ or MC$_3$ constructs demonstrated increased intracellular cAMP upon exposure to the agonists 6,7-ADTN hydrobromide or [Nle4,D-Phe7]-α-MSH, respectively, which was used to validate functional activity of the generated viral vectors (data not shown). In contrast, activation of the GHS-R1a receptor results in a Gq alpha subunit mediated increase in phospholipase C (PLC), which subsequently elevates intracellular calcium levels (Figure 5A). The intracellular calcium increase, resulting from ghrelin-mediated GHS-R1a receptor activation was reduced in cells co-expressing the D$_1$ receptor (Figure 5B). Similarly, the GHS-R1a receptor mediated intracellular calcium influx was completely blocked with MC$_3$ receptor co-expression (Figure 5C). Addition of the D$_1$ receptor agonist, 6,7-ADTN hydrobromide, or the MC$_3$ agonist, [Nle4,D-Phe7]-α-MSH, alone did not cause any significant GHS-R1a receptor mediated influx of calcium.

Functional interaction of the GHS-R1a receptor with the 5-HT$_{2C}$ receptor—Heterologous cells co-expressing the GHS-R1a receptor and the 5-HT$_{2C}$ receptor were analysed for downstream signalling consequences. Both the GHS-R1a receptor and the 5-HT$_{2C}$ receptor couple to the Gq protein, which leads to an increase in intracellular calcium. First, the EGFP and RFP constructs used for transfection and transduction were compared for their ability to elicit a calcium response in HEK293A cells (Figure 6A and B). Calcium responses were found to be independent of fluorescent tag. In addition, no 5-HT-mediated calcium response was observed in Hek-GHSR1a-EGFP cells (Figure 6A, B and C) and neither were the Hek-5-HT$_{2C}$-EGFP cells responsive in the presence of ghrelin (Figure 6A, B, D and E). Furthermore, cells co-expressing both receptors were shown not to demonstrate an additive or synergistic calcium influx when treated with a combination of 10nM agonists (5HT+ghrl) compared to when treated with either 10nM 5-HT (Figure 6D and E) or 10nM ghrelin alone (Figure 6C).

The GHS-R1a receptor has very high ligand-independent constitutive activity [61]. Reducing this constitutive activity using an inverse agonist increases receptor expression on the membrane and sensitizes receptor signalling [62, 63]. Thus, we set out to determine downstream signalling consequences following GHS-R1a receptor sensitization. To this end, GHS-R1a constitutive, ligand-independent, activity was blocked using the GHS-R1a inverse agonist, [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (SP-analog). A significant (p<0.001) ghrelin-induced enhancement of Gq-mediated
calcium influx was observed following pre-treatment with SP-analog in cells stably expressing the GHS-R1a receptor (Figure 7B), but not in cells solely expressing the 5-HT2C receptor (Figure 7A). A similar increase in GHS-R1a-mediated calcium influx was observed in cells co-expressing both the GHS-R1a receptor and the 5-HT2C receptor (Figure 7C). Interestingly, a significant (p<0.001) 5-HT induced enhancement of 5-HT2C receptor-mediated calcium influx after SP pre-treatment was observed in GHS-R1a co-expressing cell lines (Figure 7C), whereas the SP-analog was not able to increase the 5-HT induced calcium influx in cells solely expressing the 5-HT2C receptor (Figure 7A). Similar results were obtained in cells co-expressing the GHS-R1a receptor with the partially edited 5-HT2C-VSV isoform (data not shown). In addition, cells stably expressing the 5-HT2C receptor transduced to co-express the GHS-R1a receptor also demonstrated an increase in 5HT-mediated 5-HT2C receptor signalling following pretreatment with SP-analog, while absent in cells solely expressing the 5-HT2C receptor (data not shown). These results strongly suggest the potential existence of GHS-R1a-5-HT2C heterodimer.

Interestingly, we observed that the maximal calcium influx obtained with ghrelin was consistently lower in cells that co-expressed both the GHS-R1a and the 5-HT2C receptor. This suggests a 5-HT2C receptor-mediated attenuation of GHS-R1a receptor signalling (maximal relative fluorescent units obtained with FBS control for GHS-R1a/5-HT2C expressing cells (RFU 13340) was approximately 35% of that in cells expressing GHS-R1a receptor alone (RFU 36082)). To investigate this further, we analysed the ligand-mediated calcium influx relative to the ghrelin-mediated calcium response following pharmacological blockade of the 5-HT2C receptor, using RS102221 [64, 65]. Pre-treatment with RS102221 (1uM) was shown to strongly attenuate 5-HT-mediated calcium increase in heterologous cells stably expressing either the unedited 5-HT2C receptor or the partially edited 5-HT2C-VSV isoform (data not shown). A clear attenuation of MK0677- and ghrelin-induced GHS-R1a mediated signalling became apparent when comparing the ligand-mediated calcium increase in cells solely expressing the GHS-R1a receptor or co-expressing the unedited 5-HT2C receptor or the partially edited 5-HT2C-VSV isoform, (Figure 8A and B). Suppression of GHS-R1a constitutive activity by pretreatment with the GHS-R1a inverse agonist, peptide [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (1uM), increased overall GHS-R1a mediated signalling but was only able to restore attenuated GHS-R1a activation in cells co-expressing the partially edited 5-HT2C-VSV receptor isoform (Figure 8B). Full GHS-R1a activity in cells co-expressing the unedited 5-HT2C-VSV isoform was only restored following pharmacological blockade of the 5-HT2C receptor, achieved by pre-treatment with the 5-HT2C specific antagonist RS102221.

Co-expression of the edited 5-HT2C-VSV isoform was only partially able to attenuate GHS-R1a mediated signalling (Figure 8). To further investigate the potential of the 5-HT2C receptor to attenuate GHS-R1a-mediated signalling and to analyse the effect of 5-HT2C receptor editing, dose response curves were performed. Cell lines co-expressing the GHS-R1a receptor and the unedited 5-HT2C-INI receptor or the partially edited 5-HT2C-VSV receptor were pretreated with substance P-analog alone or in combination with the 5-HT2C specific antagonist, RS102221 (Figure 9). Next, calcium influx was analysed following exposure to the endogenous GHS-R1a agonist, ghrelin, or the synthetic agonist, MK0677. Pharmacological blockade of the 5-HT2C receptor had no effect on ghrelin or MK0677 mediated calcium signalling in cells that only expressed the GHS-R1a receptor (Figure 9A) or in cells co-expressing the partially edited 5-HT2C-VSV isoform in parallel to the GHS-R1a receptor (Figure 9B). However, ghrelin- and MK0677-induced calcium levels in cells co-expressing the partially edited 5-HT2C-VSV isoform were comparable to cells solely expressing the GHS-R1a receptor albeit with a slightly reduced efficacy. Cells co-expression the unedited 5-HT2C receptor isoform attenuated GHS-R1a mediated calcium signalling which was fully restored following exposure to the 5-HT2C specific antagonist, RS10221 (Figure 9C).
DISCUSSION

The realization that G protein-coupled receptors (GPCRs) do not exclusively exist or function as monomeric units has become increasingly evident from heterologous expression systems which demonstrate that GPCRs can both traffic and signal as oligomeric structures [66-69]. However, evidence for heterodimerization of centrally expressed GPCRs involved in appetite and satiety regulation is only beginning to emerge and not yet abundant. This study demonstrated promiscuous heterodimerization of the GHS-R1a receptor with both the centrally expressed MC3 receptor and the D1 receptor. Even though heterodimerization of the GHS-R1a receptor with these two GPCRs is not novel [29, 36], analysis of downstream signalling revealed a previously unreported attenuation of GHS-R1a receptor mediated calcium signalling upon co-expression compared to GHS-R1a receptor signalling alone. This may suggest a novel molecular mechanism to attenuate the GHS-R1a mediated orexigenic signalling or a dimerization-dependent modulation of the dopaminergic food reward signalling, which has recently been shown to involve the GHS-R1a receptor [15-20]. Interestingly, we show for the first time significant changes in trafficking of the GHS-R1a/D1 and GHS-R1a/MC3 receptor pairs following ligand exposure, suggesting distinct dimer-dependent mechanism in the attenuation of GHS-R1a receptor activation. Ghrelin mediated calcium signalling in the GHS-R1a/MC3 co-expressing cells is likely inhibited following attenuation of the receptor pair in the cytosol. Receptor trafficking in cells co-expressing the GHS-R1a/D1 is not affected indicating a different, yet unidentified, mechanism of GHS-R1a signalling inhibition.

Furthermore, we show evidence for a novel dimer between the GHS-R1a and the 5-HT2C receptor following ligand-mediated co-localization in cells heterologously expressing this receptor pair. We found further evidence for a GHS-R1a/5-HT2C receptor dimer when [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (SP-analog), a potent specific inverse agonist of the GHS-R1a receptor, significantly increased 5-HT2C mediated signalling in heterologous cells co-expressing the GHS-R1a/5-HT2C receptor dimer, while the SP-analog had no effect on the Gq-mediated calcium increase in cells expressing the 5-HT2C receptor alone. This inverse GHS-R1a agonist has not only been reported to suppress ligand-independent basal activity of the GHS-R1a receptor [61] but has also been shown to cause a significant increase of expression of the GHS-R1a receptor on the cell membrane, both in our lab and in reported studies [63]. Following GHS-R1a dimerization with the 5-HT2C receptor, pre-treatment with SP sensitizes the 5-HT2C receptor as well as the GHS-R1a receptor leading to an increased membrane receptor co-recruitment of the GHS-R1a/5-HT2C receptor pair. The most interesting finding was that co-expression of the unedited 5-HT2C-INI receptor, but not the partially edited 5-HT2C-VSV isoform, in cells stably expressing the GHS-R1a receptor attenuated GHS-R1a mediated intracellular calcium influx following ghrelin and MK0677 treatment. This is in line with the general consensus that increased editing of the 5-HT2C receptor leads to a decreased receptor functioning [42, 70-73]. This suggests that alteration of the 5-HT2C receptor editing profile in vivo is likely to also impact on GHS-R1a receptor signalling.

Finally, we hypothesized, that if the 5-HT2C receptor functions to attenuate the GHS-R1a mediated signalling, blockade of the 5-HT2C receptor should restore GHS-R1a mediated calcium influx. Indeed, upon 5-HT2C receptor blockade using the 5-HT2C specific antagonist, RS102221, ghrelin and MK0677-induced calcium signalling was restored in cells co-expressing the GHS-R1a receptor and the unedited 5-HT2C-INI receptor. This suggests a novel mechanism of 5-HT2C mediated attenuation of the GHS-R1a receptor. Considering the differential functional isoforms arising from 5-HT2C receptor editing this could have important biological consequences for GHS-R1a receptor function.

This is the first time, to our knowledge, that functional heterodimerization between the GHS-R1a receptor and the 5-HT2C receptor is demonstrated, suggesting that a potential relevant interaction in vivo may exist. Given that both of these GPCRs are known to regulate feeding behaviour it is tempting to
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speculate that such heterodimerization may play a role in satiety signalling. Further investigations into the extent of dimerization \textit{in vivo} are now warranted. The observed serotonin-mediated attenuation of ghrelin’s orexigenic effect in rats, as reported by Currie and colleagues, is in line with our findings and support a potential significant relevance of GHS-R1a/5-HT\textsubscript{2C} receptor dimerization in appetite regulation \textit{in vivo} [50].

Nevertheless, it is currently unclear how the intracellular calcium influx mediated by both GHS-R1a receptor and 5-HT\textsubscript{2C} receptor activation could have opposing effects on food intake \textit{in vivo}. It has been shown that ghrelin signalling can be mediated via differential tissue-specific G-protein coupling. For example, the GHS-R1a receptor has been shown to couple to G\textalpha{i2} in pancreatic islet β-cells, mediating the suppression of glucose-induced Ca\textsuperscript{2+} signalling resulting in attenuated insulin release [74]. In addition, in NPY neurons the GHS-R1a mediated Ca\textsuperscript{2+} mobilization is achieved through the Gs-cAMP-protein kinase A (PKA) signalling pathway [75]. These and other alternate downstream signalling pathways may be able to explain the opposing effects on food intake and should be further investigated.

In conclusion, these observations combined are important in strengthening the case for dimerization of the GHS-R1a receptor with several other GPCRs, including the D\textsubscript{1}, MC\textsubscript{3} and 5-HT\textsubscript{2C} receptors, and behavioural studies are now warranted to validate functional relevance of each heterodimer \textit{in vivo}. The promiscuous GHS-R1a dimerization with candidate GPCRs, such as the MC\textsubscript{3}, the D\textsubscript{1} and the 5-HT\textsubscript{2C} receptor may play a key role in the modulation and fine-tuning of GHS-R1a mediated downstream signalling and subsequent satiety and appetite signalling as well as the reward and motivational aspects of food intake. The implications of GHS-R1a receptor heterodimerization will fundamentally impact the current knowledge on the structure, activation and desensitization processes of this receptor. In addition, novel heterodimerization of GPCRs involved in energy homeostasis and reward as well as further elucidating of the downstream signalling pathways will lead to a better understanding of not only homeostatic regulation of food intake but also the incentive motivational value and rewarding aspects of food. This may ultimately have dramatic impacts on drug development and screening and lead to novel ghrelin targeted therapies with increased selectivity and reduced side effects. Heterodimer complexes of the GHS-R1a receptor provide unique pharmacological targets to control a subset of functions within a broader ghrelin-mediated signalling spectrum of feeding behaviours, including homeostatic appetite signalling as well as the hedonic pathways in response to food and drugs of abuse. The potential role for the GHS-R1a/5-HT\textsubscript{2C} dimer in the ghrelin-mediated effects on the rewarding properties of food warrants further investigation.
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FOOTNOTES
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FIGURE LEGEND

Figure 1 Colocalization of the GHS-R1a receptor with candidate GPCRs in Hek293A cells. Hek293A cells stably expressing the GHS-R1a receptor as an EGFP fusion protein were transduced with lentiviral vectors expressing candidate GPCRs as RFP fusion proteins. Colocalization of fluorescence was analyzed using confocal microscope, and is indicated by yellow colour overlap. A) Colocalization (yellow) of the D1 receptor (red) with the GHS-R1a receptor (green) was observed. B) Colocalization of the MC3 receptor (red) with the GHS-R1a (green) receptor was only observed intracellular. C) Colocalization of the 5-HT2C receptor (red) with the GHS-R1a receptor (green) was demonstrated both intracellular and on the membrane. D) The edited 5-HT2C isoform, 5-HT2C-VSV receptor (red), also demonstrated colocalization with the GHS-R1a receptor mainly in the intracellular space.

Figure 2 Co-internalization of the GHS-R1 receptor with the D1 receptor. Hek293A cells stably expressing GHS-R1a-EGFP were transduced with viral vectors expressing lvDR1-RFP. Successful expression of the D1 receptor (red), the GHS-R1a receptor (green) and colocalization (yellow) could be observed 48 hours after transduction (A, F, K). Cells were incubated for 60 minutes with 10μM of the specific D1 receptor agonist, 6,7-ADTN hydrobromide (B, G, L), with 1μM ghrelin (C, H, M), with 1μM MK0677 (D, I, N) or with 1μM of [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (E, J, O). Images were made using a CKX41 inverted microscope (Olympus) with a XM10 camera and CellFv3.3 software. Quantified agonist-mediated co-internalization is depicted (P). Statistical significance was analysed using analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; statistical significance of agonist-mediated co-internalization compared to control is notated as ***p<0.001, **p<0.01 and *p<0.05. Statistical significant difference compared to [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P is notated as ^^^p<0.001, ^^p<0.01 and ^p<0.05.

Figure 3 Co-internalization of the GHS-R1 receptor with the MC3 receptor. Hek293A cells stably expressing GHS-R1a-EGFP were transduced with viral vectors expressing lvMC3-RFP. Successful expression or the MC3 receptor (red), the GHS-R1a receptor (green) and colocalization (yellow) could be observed 48 hours after transduction (A, F, K). Cells were incubated for 60 minutes with 10μM of the specific MC3-agonist, [Nle4,D-Phe7]-α-MSH (B, G, L), with 1μM ghrelin (C, H, M), with 1μM MK0677 (D, I, N) or with 1μM of [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (E, J, O). Images were made using a CKX41 inverted microscope (Olympus) with a XM10 camera and CellFv3.3 software. Quantified agonist-mediated co-internalization is depicted (P). Statistical significance was analysed using analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; statistical significance of agonist-mediated co-internalization compared to control is notated as ***p<0.001, **p<0.01 and *p<0.05. Statistical significant difference compared to [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P is notated as ^^^p<0.001, ^^p<0.01 and ^p<0.05.

Figure 4 Co-internalization of the GHS-R1 receptor with the 5-HT2C receptor. Hek293A cells stably expressing GHS-R1a-EGFP were transduced with viral vectors expressing lv5-HT2C-RFP. Successful expression or the 5-HT2C receptor (red), the GHS-R1a receptor (green) and colocalization (yellow) could be observed 48 hours after transduction (A, F, K). Cells were incubated for 60 minutes with 100nM of serotonin (5-HT) (B, G, L), with 1μM ghrelin (C, H, M), with 100nM MK0677 (D, I, 14
N) or with 1uM of [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (E, J, O). Images were made using a CKX41 inverted microscope (Olympus) with a XM10 camera and Cell^Fv3.3 software. Quantified agonist-mediated co-internalization is depicted (P). Statistical significance was analysed using analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; statistical significance of agonist-mediated co-internalization compared to control is notated as ***p<0.001, **p<0.01 and *p<0.05. Statistical significant difference compared to [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P is notated as ^^^p<0.001, ^^p<0.01 and ^p<0.05.

**Figure 5 Co-expression of D₁ or MC₃ attenuates GHS-R1a mediated signalling in Hek293A cells.** Ghrelin-mediated intracellular calcium increase (A) is significantly reduced when the D₁ receptor (B) or the MC₃ receptor (C) are co-expressed with the GHS-R1a receptor in hek293A cells. The cells were incubated for 30 minutes with 100nM ghrelin, 10uM 6,7-ADTN hydrobromide or [Nle4,D-Phe7]-α-MSH or a combination of both and intracellular calcium influx was measured. Intracellular calcium increase was depicted as a percentage of maximal calcium increase as elicited by control (3% FBS). The data is a representative figure from three independent experiments depicting the mean ±SEM with each concentration point performed in triplicate. Statistical significance was analysed using a two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; statistical significance is notated as ***p<0.001 and **p<0.01.

**Figure 6 No synergistic or additive effects on intracellular calcium signalling in cells co-expressing the GHS-R1a and the 5-HT₂C receptor.** Co-expression of the GHSR1a receptor (A) or the 5-HT₂C receptor (B) as EGFP or RFP fusion proteins yields similar ligand-mediated calcium increases. No synergistic or additive effects are observed in cells co-expressing the GHS-R1a receptor and the 5-HT₂C-RFP receptor (C), or cells stably expressing the unedited 5-HT₂C-INI (D) or the partially edited 5-HT₂C-VSV (E) receptor transduced with the GHS-R1a-RFP receptor, following treatment with 10nM or 100nM 5HT (light gray bars), 10nM or 100nM ghrl (dark gray bars) or with a combination of 10nM both (black bar). The data represents the mean ±SEM of an experiment performed in triplicate for each concentration point with the intracellular calcium increase depicted as a percentage of maximal calcium increase as elicited by control (3% FBS). Statistical significance within the 10nM datasets was analysed using two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; Statistical significant effects of combination treatment (10nM serotonin, 5-HT+ 10nM ghrelin, ghrl) were compared to 10nM 5-HT alone and notated as “a” p<0.01 or to 10nM ghrl alone and notated as “b” p<0.001.

**Figure 7 Enhanced 5-HT₂C mediated signalling when GHS-R1a ligand-independent activity is blocked.** Cells stably expressing the GHS-R1a receptor were transduced with viral vectors expressing the 5-HT₂C receptor and treated with 100nM 5HT (light gray bars), 100nM ghrl (dark gray bars) with or without pre-treatment of GHS-R1a inverse agonist, peptide [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (1uM) and intracellular calcium influx was measured. Substance P (SP) has no effect on 5-HT₂C mediated signalling (A) but a significant increase in GHS-R1a mediated Gq activation is observed following SP pre-treatment (B). Interestingly, 5-HT₂C receptor mediated calcium influx is enhanced after SP pre-treatment when the GHS-R1a receptor is co-expressed (C). Intracellular calcium increase is depicted in relative fluorescence units (RFU) as a percentage of maximal calcium increase as elicited by control in each separate experiment (3% FBS). The data represents the mean ±SEM of two independent experiments performed in triplicate for each concentration point. Analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; statistical significance is notated as a=p<0.001, 5-HT compared to ghrl; b=p<0.001, 5-HT+SP compared to ghrl+SP; ***p<0.001, treatment with SP compared to no SP.

**Figure 8 Co-expression of the 5-HT₂C receptor attenuates GHS-R1a mediated signalling.** Cells stably expressing the GHS-R1a receptor (light gray bars) were transduced with viral vectors expressing the 5-HT₂C receptor (dark gray bars) or the partially edited isoform, 5-HT₂C-VSV (striated bars) and treated with 100nM ghrl or 100nM MK0677 and with or without (1uM) RS10221 exposure. Intracellular calcium increase was reduced in cells co-expressing the 5-HT₂C receptor which was rescued after RS102221 treatment (A). The GHS-R1a inverse agonist, peptide [D-Arg1, D-Phe5,D-Trp7,9, Leu11]-substance P (1uM), increased total calcium influx but was not able to restore full
GHS-R1a mediated signalling (B). Intracellular calcium increase was depicted in relative fluorescence units (RFU) as a percentage of maximal calcium increase as elicited by ghrelin or MK0677 response in presence of RS102221. Intracellular calcium increase was depicted as a percentage of maximal calcium increase as elicited by control. The data represents the mean ±SEM of a representative experiment out of 3 independent transductions with each concentration point performed in triplicate. Two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test; statistical significance is notated as ***p<0.001 and **p<0.01.

**Figure 9 Attenuation of GHS-R1a mediated signalling depends on specific 5-HT$_{2C}$ isoform co-expression.** Cells co-expressing the GHS-R1a receptor and the 5-HT$_{2C}$ receptor or the partially edited isoform, 5-HT$_{2C}$-VSV were treated with ghrl or MK0677 in a dose respond manner (300nm - 3.7nM, 3 fold serial dilution), following pre-treatment with the GHS-R1a inverse agonist, peptide [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (1uM). No effect of 5-HT$_{2C}$ receptor blockade in cells solely expression the GHS-R1a receptor (A). RS102221 rescues GHS-R1a induced calcium influx in GHS-R1/5-HT$_{2C}$ expressing cells (B) but no change in GHS-R1a/5-HT$_{2C}$-VSV expressing cells (C). Intracellular calcium increase was depicted as a percentage of maximal calcium increase as elicited by control in each separate experiment (3% FBS in presence of SP-analog). The data represents the mean ±SEM of a representative experiment out of 3 independent transductions with each concentration point performed in triplicate.
FIGURE 1
A novel heterodimer of the GHS-R1a and the 5-HT$_{2C}$ receptor

FIGURE 2
A novel heterodimer of the GHS-R1α and the 5-HT<sub>2C</sub> receptor
A novel heterodimer of the GHS-R1a and the 5-HT₂C receptor
A novel heterodimer of the GHS-R1a and the 5-HT₂C receptor
A novel heterodimer of the GHS-R1a and the 5-HT$_{2C}$ receptor

FIGURE 6
FIGURE 7

A novel heterodimer of the GHS-R1a and the 5-HT\textsubscript{2C} receptor
A novel heterodimer of the GHS-R1a and the 5-HT$_{2C}$ receptor

FIGURE 8

A

B
FIGURE 9
Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signalling
Harriet Schellekens, Wesley E.P.A. van Oeffelen, Timothy G. Dinan and John F. Cryan
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