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Ghrelin's Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction

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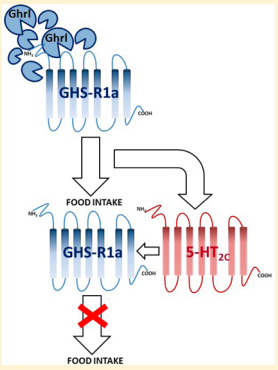
ABSTRACT: Understanding the intricate pathways that modulate appetite and subsequent food intake is of particular importance considering the rise in the incidence of obesity across the globe. The serotonergic system, specifically the 5-HT_{2C} receptor, has been shown to be of critical importance in the regulation of appetite and satiety. The GHS-R1a receptor is another key receptor that is 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 signaling is not due to coupling to $G\alpha_S$, as no increase in cAMP signaling is observed. Next, flow cytometry 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 colocalized 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 signaling 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 a biologically 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.



KEYWORDS: *Ghrelin, growth hormone secretagogue receptor, serotonin 2C receptor, lorcaserin, food intake*

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 antiobesity 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 ref 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 5-HT_{2C} receptors.[27]

Interestingly, serotonergic signaling has long been known to be involved in controlling food intake and to impact satiety.[28−38] Individuals with normally 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 (*m*CPP).[29,37,41,42] Unfortunately, none of these drugs have been without heart and pulmonary

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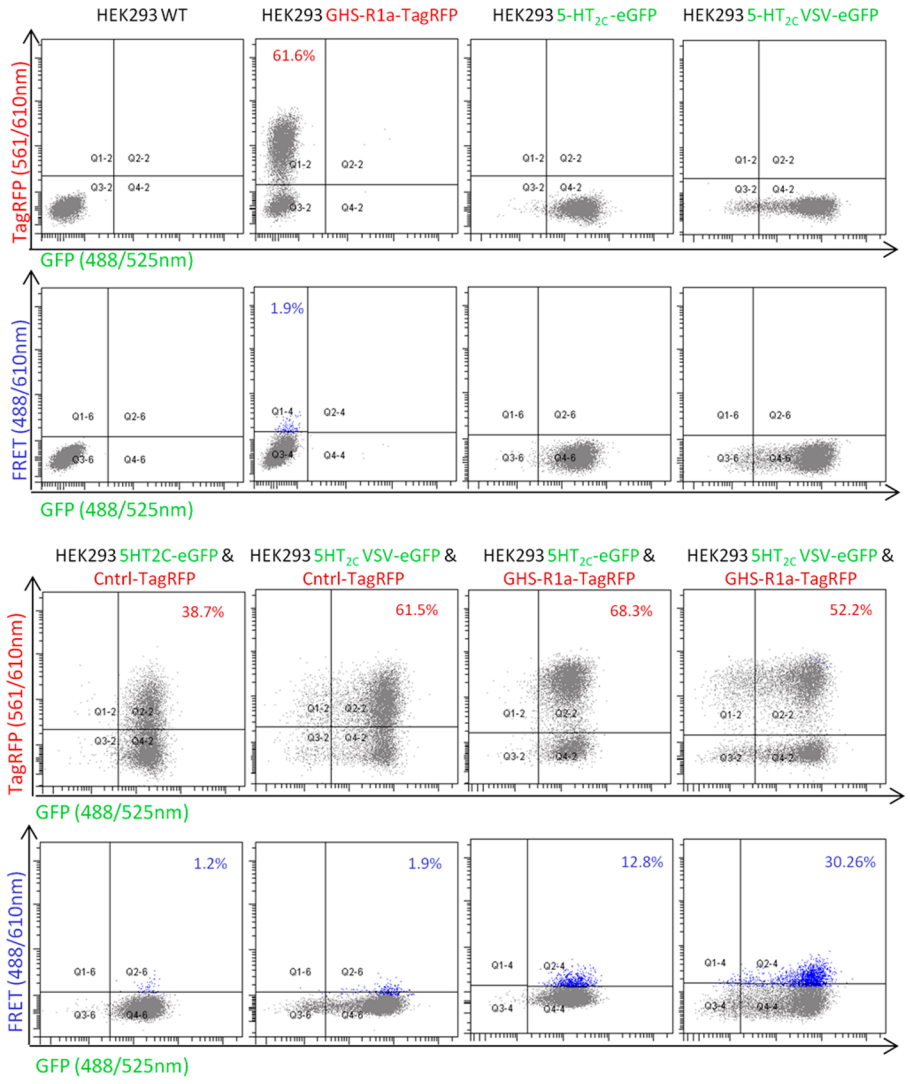

Figure 1. FRET between the 5-HT$_{2C}$ and GHS-R1a receptors. Hek293A cells stably expressing the 5-HT$_{2C}$ receptor as an eGFP fusion protein or the partially edited 5-HT$_{2C}$ isoform, 5-HT$_{2C}$-VSV-eGFP, were transiently transduced with lentiviral vectors expressing control-TagRFP or GHS-R1a-TagRFP. Cells were analyzed 72 h 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).

vasculature side effects or have been associated with a poor efficacy and other nonspecific 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 in food intake, 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 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 signaling 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 signaling 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$_{2C}$ receptors *in vitro*,[27] demonstrating attenuated GHS-R1a signaling following coexpression of the 5-HT$_{2C}$ receptor, which reinforces the physiological relevance of the GHS-R1a/5-HT$_{2C}$ dimer.

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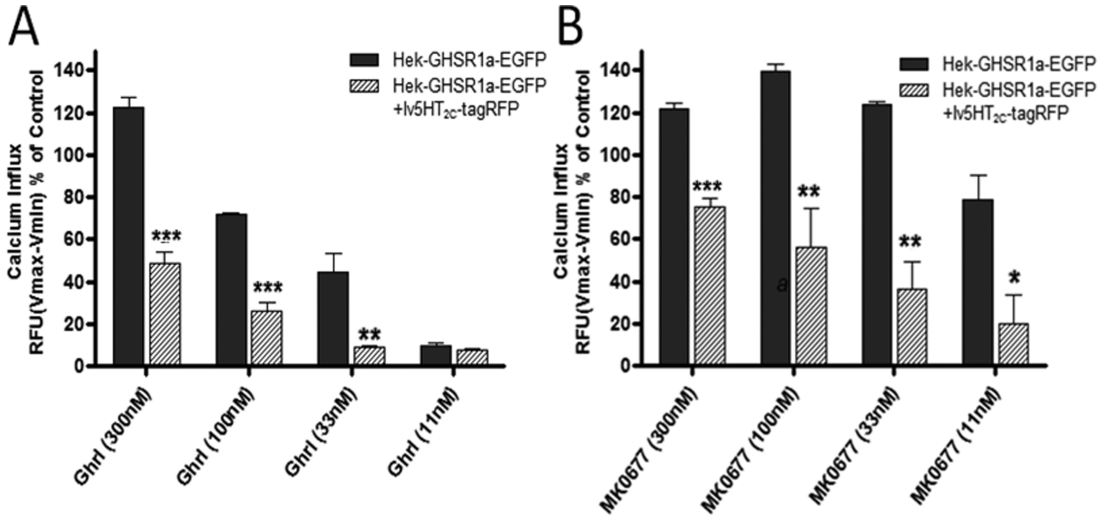

Figure 2. Coexpression of the 5-HT$_{2C}$ receptor attenuates GHS-R1a-mediated intracellular calcium mobilization. The ligand-mediated intracellular calcium increase in Hek293A cells stably expressing the GHS-R1a receptor only (solid bars) was reduced when coexpressing the 5-HT$_{2C}$ receptor (striated bars) following exposure to different concentrations of ghrelin (A) or different concentrations of the synthetic agonist, MK0677 (B). Intracellular calcium mobilization is 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 that in cells solely expressing the GHS-R1a receptor is denoted as *, indicating $p < 0.05$; **, indicating $p < 0.01$; or ***, indicating $p < 0.001$.

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$_{2C}$ receptors in relation to their function in appetite, and we analyze the significance of the interaction of these two key receptors *in vivo*. Specifically, the colocalized expression of endogenous levels of these receptors in neuronal cultures is investigated using a recently described fluorescein-labeled ghrelin peptide tracer.[58,59] Finally, the effects of specific 5-HT$_{2C}$ receptor antagonism versus agonism on ghrelin's orexigenic effect is analyzed 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 behavior *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 Coexpression 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 (fcFRET). 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 five specific nucleotide positions (A, B, C, D, E) in the second 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 behavior and fat mass.[38,66,68] Noteworthy increases in FRET levels, as a percentage of tagRFP expression, were observed 72 h post-transduction (Figure 1). Following lentiviral transduction of Hek293 cells with the lvGHS-R1a-tagRFP vector, 61.6% of cells were analyzed as positive for tagRFP expression (Figure 1, first row, column 2), with relatively no FRET signal (1.6%, Figure 1, second 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, first and second rows). 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, third row, columns 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 Hek 5-HT$_{2C}$-VSV-eGFP cells, respectively (Figure 1, third row, columns 3 and 4). Finally, when analyzing flow cytometry fluorescence energy transfer as a measure of heterodimerization, coexpression 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 that with control-TagRFP vectors, respectively (Figure 1, fourth row). These significant increases in FRET signal are further evidence of a physical interaction between the GHS-R1a and 5-HT$_{2C}$ receptors. Interestingly, we consistently found a >2× higher percentage of FRET signal when the GHS-R1a receptor is coexpressed with the edited 5-HT$_{2C}$-VSV variant of the receptor compared to that with the fully unedited 5-HT$_{2C}$ receptor. This may suggest that 5-HT$_{2C}$ receptor editing can modulate dimer formation and warrants further investigations.

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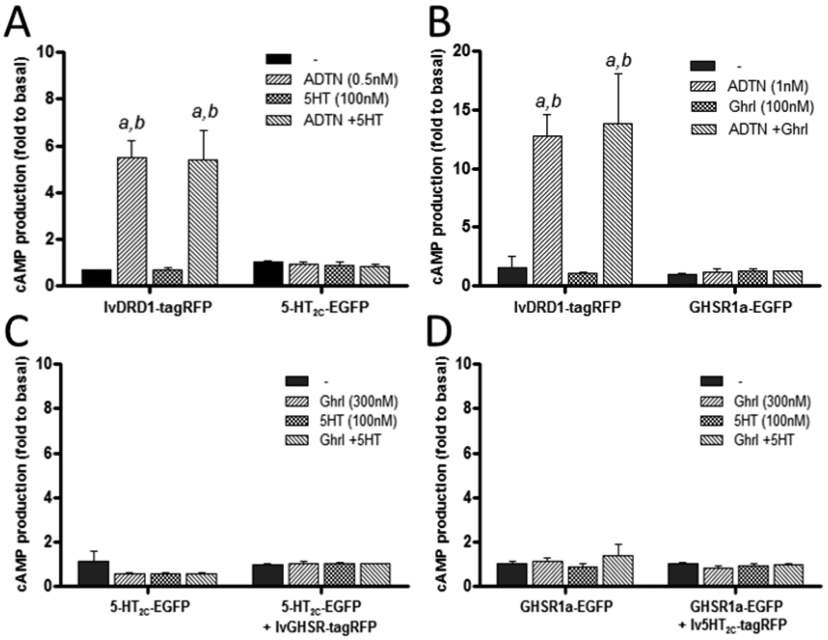

Figure 3. Coexpression of the 5-HT_{2C} and GHS-R1a receptors does not influence cAMP signaling. The dopamine D_1 receptor agonist, 6,7-ADTN hydrobromide (0.5 nM), induces an increase in cAMP in human embryonic cells transiently expressing the D_1 receptor following lentiviral transduction (lvDRD1-tagRFP) but not in cells stably expressing the 5-HT_{2C} receptor (A) or the GHS-R1a receptor (B). Coexpression 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). Lentiviral coexpression of 5-HT_{2C} receptor (lv5HT_{2C}-EGFP) in cells stably expressing the GHS-R1a receptor does not induce intracellular cAMP production (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 *a*, $p < 0.001$ compared to vehicle (−), and *b*, $p < 0.001$ compared to 5-HT (A) or ghrelin (Ghrl) (B), respectively.

Intracellular Signalling upon Coexpression of the GHS-R1a Receptor with the 5-HT_{2C} Receptor. 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 analyzed ligand-mediated downstream signaling consequences following coexpression of fluorescently tagged receptors. To this end, heterologous cells coexpressing the GHS-R1a-EGFP and 5-HT_{2C}-RFP receptors were analyzed 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,[27] was reduced when coexpressing the 5-HT_{2C} receptor (Figure 2A). In addition, a similar attenuation of the GHS-R1a-mediated intracellular calcium mobilization upon coexpression of the 5-HT_{2C} receptor was observed when the synthetic GHS-R1a ligand, MK0677, was used (Figure 2B). This is in line with our previous study and confirms the 5-HT_{2C} receptor-mediated attenuation of GHS-R1a receptor signaling, 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 enhanced dopamine-induced cAMP accumulation[24] and attenuated GHS-R1a-mediated calcium signaling.[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 coexpressing both the GHS-R1a and 5-HT_{2C} receptors (Figure 3). First, we analyzed Hek293 cells transduced with the D_1 receptor expressing vectors (lvDRD1-tagRFP) as positive controls (Figure 3A,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 (0.5 nM), 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 (100 nM) or ghrelin (100 nM) exposure (Figure 3A,B). In addition, no ligand-mediated cAMP responses were observed in Hek293 cells stably expressing the 5-HT_{2C}-eGFP receptor (Figure 3A,C) or in 5-HT_{2C}-expressing cells transduced with lvGHS-R1a-tagRFP vectors (Figure 3C). Moreover, no ligand-mediated cAMP response were observed in Hek-GHS-R1a-EGFP cells (Figure 3B,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 coexpressing the GHS-R1a receptor with the partially edited 5-HT_{2C}-VSV isoform (data not shown). Thus, coexpression 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.

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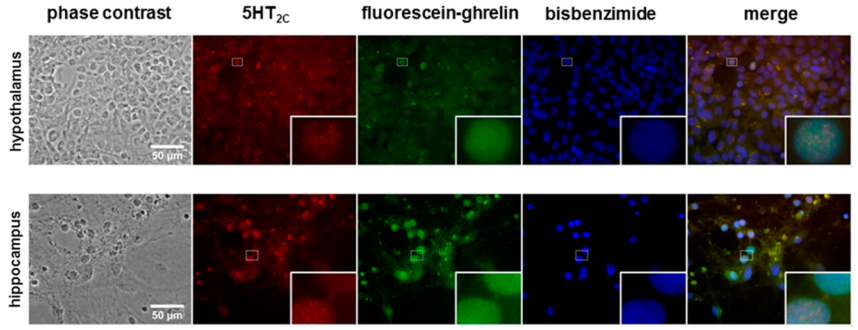

Figure 4. Colocalization of the 5-HT_{2C} receptor and ghrelin-fluorescein staining in rat hippocampal and hypothalamic neurons. Primary cultured hypothalamic (top panel) and hippocampal (bottom panel) cells were shown to express the 5-HT_{2C} receptor, indicated in red, and also to bind fluorescein-ghrelin, 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).

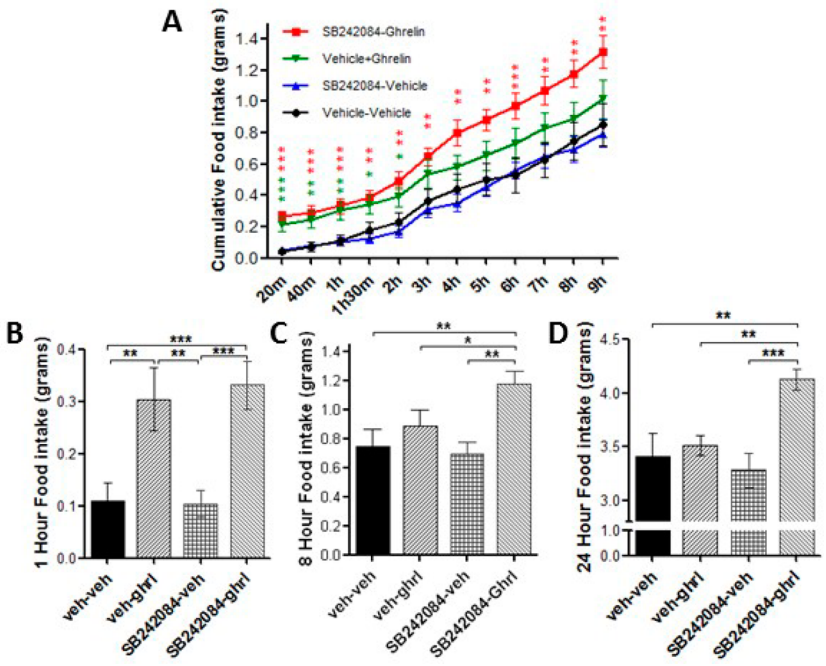

Figure 5. Specific 5-HT_{2C} receptor antagonism potentiates ghrelin's orexigenic effect *in vivo*. Cumulative food intake (A) and food intake at time points at 1, 8, and 24 h (B, C, D) are depicted for *ad libitum* fed male C57BL/6 mice following intraperitoneal administration of the brain-penetrant 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. Statistically 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.

Colocalization of the 5-HT_{2C} Receptor and Fluorescein-Ghrelin Binding *ex Vivo*. Next, endogenous coexpression of the GHS-R1a and 5-HT_{2C} receptors 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. demonstrated 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 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, as well as on the POMC neurons, albeit only in 8% of the POMC neurons.[80] Here, we investigated the colocalization 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 analyzed using a variation of a recently described method using fluorescein-ghrelin,[82] a novel strategy to detect specific GHS-R1a receptor expression.[58] Colocalization of the 5-HT_{2C} receptor and fluorescein-ghrelin binding was correlated in

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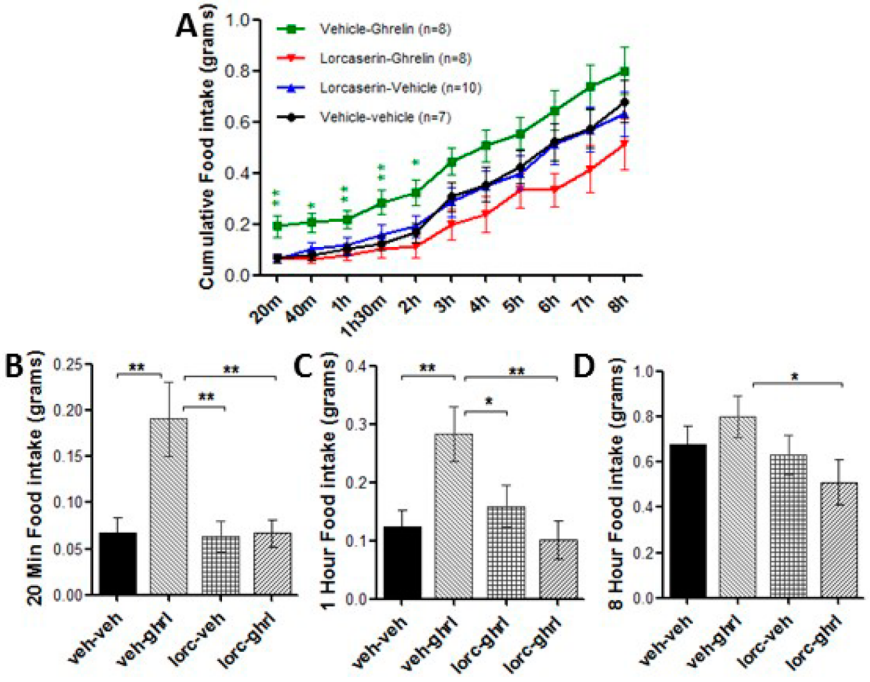

Figure 6. Specific 5-HT_{2C} receptor agonism attenuates ghrelin's orexigenic effect *in vivo*. Cumulative food intake (A) and food intake at time points 20 min, 1 h, and 8 h (B, C, D) are depicted for *ad libitum* fed male C57BL/6 mice following subcutaneous administration of the 5-HT_{2C}-specific 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. Statistically 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.

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 coexpressed both receptors. In the hippocampus, both receptors were expressed at higher levels, and cells expressing only one receptor were more frequently found. Indeed, the inset in the bottom picture shows two cells that are both positive for fluorescein-ghrelin binding to the GHS-R1a receptor, but one of them is lacking staining for the 5-HT_{2C} receptor (Figure 4, bottom panel). This data clearly demonstrates the colocalized endogenous expression of the GHS-R1a and 5-HT_{2C} receptors, which is the 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 analyzed the effect of specific 5-HT_{2C} receptor antagonism on ghrelin's orexigenic potential *in vivo*. Food intake of male C57BL/6 mice was analyzed 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 ($F(6.1,46.932) = 3.817$; $p = 0.003$). Posthoc analysis of the cumulative food intake indicated that the significance of ghrelin's orexigenic effect compared to vehicle tapers off after the 2 h 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 that is diminished over time.[4,83] Interestingly, the significance of the ghrelin-induced increase in food intake was maintained after the 2 h time point following SB242084-mediated 5-HT_{2C} receptor antagonism ($p < 0.01$), resulting in a ghrelin-mediated increase in food intake that was still apparent at 9 h, whereas 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 (Figure 2 and see ref 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 coadministration, compared to that with ghrelin alone, are individually depicted in bar graphs and clearly visible at 8 and 24 h after food placement but not at 1 h (Figure 5B,C,D). At the 1 h time point, ghrelin's effect is still significant compared to control, and coadministration 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 analyzed 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 analyzed (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

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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 that with vehicle, which lasted up to 2 h, after which significance tapers off (Figure 6A–D). Interestingly, ghrelin's initial orexigenic effect was not observed when animals also received the 5-HT_{2C}-specific agonist, lorcaserin, at 3 mg/kg. Indeed, when the 5-HT_{2C} receptor is activated using lorcaserin, the acute orexigenic effect is completely blocked in the first 2 h. Furthermore, no effect on food intake was observed with this subthreshold dose of lorcaserin on its own (Figure 6A). At the 8 h time point, ghrelin's orexigenic effect compared to control is no longer observed, but the combination treatment actually has a significantly decreased food intake compared to ghrelin, reinforcing the significant inhibition of 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 signaling, in line with previous findings.[27,55] 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 behavior. Indeed, recent studies have identified the ghrelinergic system as a key player in hedonic food intake behaviors, 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 behaviors,[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 that 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 inhibitory control on dopaminergic neurons in the ventral tegmental area (VTA) through the activation of GABAergic interneurons (for review, see ref 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 signaling through the mesolimbic pathway are now warranted.

CONCLUSIONS

Together, this study shows compelling evidence for a functionally relevant interaction between the GHS-R1a and 5-HT_{2C} receptors. 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 GHS-R1a *in vitro* when the 5-HT_{2C} receptor is coexpressed (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 signaling 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} receptors 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 and 5-HT_{2C} receptors 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 nonpeptide 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, no. 1946; Tocris) were prepared at a 1 mM stock solution in assay buffer, consisting of 1× Hanks balanced salt solution (HBSS) supplemented with 20 mM 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 signaling 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 (no. 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) 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 fetal 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 GHS-R1a-EGFP, 5-HT_{2C}-EGFP, or 5-HT_{2C}-VSV-EGFP were transduced using lentiviral vectors to coexpress 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 8 μg/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 coexpression of the 5-HT_{2C}-RFP construct following lentiviral transduction (data not shown). All cell lines were generated

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following approval and in full accordance with the Environmental Protection Agency (Ireland) under GMO register no. G0331-01.

Flow Cytometry Fluorescence Resonance Energy Transfer (fcFRET). Cells were harvested 48 to 72 h after transduction using 37 °C trypsin/EDTA, centrifuged, resuspended in PBS, and passed through a cell strainer with 40 μm nylon mesh (BD Biosciences, no. 352340) prior to analysis. fcFRET analysis was performed on an LSR II cytometer (BD biosciences), and eGFP was excited at 488 nm and detected with a 525/50 nm bandpass filter, whereas TagRFP was excited at 561 nm and detected with a 610/20 nm bandpass filter. FRET between eGFP and TagRFP was measured by excitation at 488 nm 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 analyzed. Live cells were gated according to forward and side scattering (FSC/SSC). Nontransduced 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 analyzed using FACSDiva software (BD Biosciences).

Calcium Mobilization Assay. Receptor-mediated changes in intracellular calcium (Ca^{2+}) were analyzed as previously described.[27] Briefly, stably transfected cells were seeded in black 96-well microtiter plates at a density of 2.5×10^5 cells/mL (2.5×10^4 cells/well) and maintained for ∼24 h at 37 °C in a humidified atmosphere containing 5% CO_2. On the day of the assay, growth medium was aspirated, and cells were incubated with 25 μL of assay buffer (1× Hanks balanced salt solution, HBSS, supplemented with 20 mM HEPES buffer) and 25 μL of Calcium 4 dye (R8141, Molecular Devices Corporation, Sunnyvale, CA) according to the manufacturer's protocol. Cells were pretreated with 1 μM of the GHS-R1a inverse agonist, peptide [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (no. 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 s in flex mode with an 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 ($V_{max} - V_{min}$; 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 analyzed 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; no. TRF0262), according to the manufacturer's instructions. Briefly, 5 μL of a 2×10^5 cells/mL suspension was plated per well in a white 384-well plate (PerkinElmer; Optiplate 6007291). Receptor activation was stimulated via the addition of 5 μL per well of the D_1 receptor agonist, 6,7-ADTN hydrobromide (Ascent Scientific; Asc-150). Following 30 min incubation at room temperature, 5 μL per well of Eu-cAMP tracer in stimulation buffer and 5 μL per well of monoclonal Ulight-anti-cAMP antibody were added and incubated for 1 h 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 were measured at 615 and 665 nm in the Synergy 2 multimode microplate reader (BioTek). A quench correction was performed to minimize false positives and negatives by calculating the blank corrected ratio 665 nm/615 nm using the equation $F665,CS = [(F665,S - F665,BL) \times F615,MAX]/F615,S$. The blank value is separately measured by adding buffer to the wells to obtain blank reading at 665 nm. Data was analyzed 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 ventral side up. Microdissection forceps were 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 hemispheres. 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. Afterward, cells were dissociated with a solution containing trypsin 0.25 mg/mL (cat. no. L2700-100, Microvet) and deoxyribonuclease I from bovine pancreas 0.28 mg/mL (cat. no. D5025, Sigma-Aldrich) at 37 °C for 20 min, 300 μL of FBS was then added to stop the digestion, and cells were mechanically dissociated using several glass pipettes with consecutive smaller tip diameters. Cells were seeded on 24 × 24 mm glass (5×10^4 cells/each) previously treated with poly-L-lysine (cat. no. 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. no. 21051-016, Gibco), 3.3 μg/mL insulin (Nordisk Pharm Ind, Inc., Clayton, NC, USA), 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. no. 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. no. 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.[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 with 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% Triton X in PBS), incubated with goat anti-fluorescein antibody conjugated to Alexa Fluor 488 (Molecular Probes, A-11096, 1:100 in blocking solution) for 2 days at 4 °C, and washed with PBS. Afterward, cells were incubated with mouse anti-5-HT_{2C} antibody (Santa Cruz, cat. no. sc-17797, 1:200 in blocking solution) for 24 h at 4 °C, washed with PBS, and finally incubated for 1 h at room temperature with donkey anti-mouse antibody conjugated to Alexa Fluor 594 (Molecular Probes, cat. no. A21203, 1:1000 in blocking solution) and rinsed with PBS. Negative controls were generated by omitting the primary or secondary antibody, 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 four to a standard holding cage at the animal care facility

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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.[83,102] Briefly, the mice were weighed, single-housed in new cages in the experimental room, and habituated for 20 min 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 was used. For the first injection, SB242084 (no. 2901; Tocris) (2.0 mg/kg in saline and 1.0% DMSO) and vehicle (saline with 1.0% DMSO) were administered, 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 was 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 h food restriction (data not shown). The subthreshold dose of 3 mg/kg (0.3 mg/mL) was selected for further experiments because no effect on food intake was observed using this dose for up to 8 h. For the 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 of ghrelin (Innovagen; SP-GHRL-1) (200 nmol/kg in saline) and vehicle (saline) via intraperitoneal (IP) administration (10 μL/gram of body weight). The time between the first and second injections was 15 min, and preweighed chow food pellets were carefully placed in the experimental cages 20 min following the second IP injection. Thereafter, the amount of food was weighed at regular time intervals (20 min, 40 min, 1 h, 1 h 30 min, 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 analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.). All experiments were conducted in accordance with the European Directive 86/609/EEC, 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, USA). For *in vitro* assays, significance was determined by 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 posthoc test for each time point. If the data was nonspherical, then a Huynh−Feldt correction was applied. Graphs were expressed as the 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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Author Contributions

H.S. designed all experiments with the exception of the experiments that lead to Figure 4. T.M. performed the experiments that lead to Figure 1. H.S. performed experiments that lead to Figure 2 and 3. P.N.D.F. performed the experiments that lead to Figure 4 in the laboratory of M.P., who designed the experiment. W.F.T. performed the experiment that lead to Figure 5. W.E.P.A.O and D.K. performed the experiments that lead to Figure 5. H.S. wrote the manuscript. L.G. and M.P. critically read the manuscript and T.G.D. and J.F.C. offered scientific guidance throughout.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature 402*, 656−660.

(2) Davenport, A. P., Bonner, T. I., Foord, S. M., Harmar, A. J., Neubig, R. R., Pin, J. P., Spedding, M., Kojima, M., and Kangawa, K. (2005) International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. *Pharmacol. Rev. 57*, 541−546.

(3) Andrews, Z. B. (2011) Central mechanisms involved in the orexigenic actions of ghrelin. *Peptides 32*, 2248−2255.

(4) Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001) A role for ghrelin in the central regulation of feeding. *Nature 409*, 194−198.

(5) Tschop, M., Smiley, D. L., and Heiman, M. L. (2000) Ghrelin induces adiposity in rodents. *Nature 407*, 908−913.

(6) Kojima, M., Hosoda, H., and Kangawa, K. (2004) Clinical endocrinology and metabolism. Ghrelin, a novel growth-hormone-releasing and appetite-stimulating peptide from stomach. *Best Pract. Res. 18*, 517−530.

(7) Yi, C. X., Heppner, K., and Tschop, M. H. (2011) Ghrelin in eating disorders. *Mol. Cell. Endocrinol. 340*, 29−34.

(8) Patterson, M., Bloom, S. R., and Gardiner, J. V. (2011) Ghrelin and appetite control in humans—potential application in the treatment of obesity. *Peptides 32*, 2290−2294.

(9) Schellekens, H., Dinan, T. G., and Cryan, J. F. (2009) Lean mean fat reducing "ghrelin" machine: hypothalamic ghrelin and ghrelin receptors as therapeutic targets in obesity. *Neuropharmacology 58*, 2−16.

(10) Horvath, T. L., Castaneda, T., Tang-Christensen, M., Pagotto, U., and Tschop, M. H. (2003) Ghrelin as a potential anti-obesity target. *Curr. Pharm. Des 9*, 1383−1395.

(11) Zorrilla, E. P., Iwasaki, S., Moss, J. A., Chang, J., Otsuji, J., Inoue, K., Meijler, M. M., and Janda, K. D. (2006) Vaccination against weight gain. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13226−13231.

(12) Soares, J. B., Roncon-Albuquerque, R., Jr., and Leite-Moreira, A. (2008) Ghrelin and ghrelin receptor inhibitors: agents in the treatment of obesity. *Expert Opin. Ther. Targets 12*, 1177−1189.

(13) Moulin, A., Ryan, J., Martinez, J., and Fehrentz, J. A. (2007) Recent developments in ghrelin receptor ligands. *ChemMedChem 2*, 1242−1259.

DOI: 10.1021/cn500318q
ACS Chem. Neurosci. 2015, 6, 1186−1197

(14) Lu, S. C., Xu, J., Chinookoswong, N., Liu, S., Steavenson, S., Gegg, C., Brankow, D., Lindberg, R., Veniant, M., and Gu, W. (2009) An acyl-ghrelin-specific neutralizing antibody inhibits the acute ghrelin-mediated orexigenic effects in mice. *Mol. Pharmacol. 75*, 901−907.
(15) Leite-Moreira, A. F., and Soares, J. B. (2007) Physiological, pathological and potential therapeutic roles of ghrelin. *Drug Discovery Today 12*, 276−288.
(16) Chollet, C., Meyer, K., and Beck-Sickinger, A. G. (2009) Ghrelin—a novel generation of anti-obesity drug: design, pharmaco-modulation and biological activity of ghrelin analogues. *J. Pept. Sci. 15*, 711−730.
(17) Holst, B., Brandt, E., Bach, A., Heding, A., and Schwartz, T. W. (2005) Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling. *Mol. Endocrinol. 19*, 2400−2411.
(18) Leung, P. K., Chow, K. B., Lau, P. N., Chu, K. M., Chan, C. B., Cheng, C. H., and Wise, H. (2007) The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor. *Cell Signal 19*, 1011−1022.
(19) Schellekens, H., Dinan, T. G., and Cryan, J. F. (2013) Taking two to tango: a role for ghrelin receptor heterodimerization in stress and reward. *Front. Neurosci. 7*, 148.
(20) Chan, C. B., and Cheng, C. H. (2004) Identification and functional characterization of two alternatively spliced growth hormone secretagogue receptor transcripts from the pituitary of black seabream *Acanthopagrus schlegeli*. *Mol. Cell. Endocrinol. 214*, 81−95.
(21) Chow, K. B., Sun, J., Chu, K. M., Tai Cheung, W., Cheng, C. H., and Wise, H. (2012) The truncated ghrelin receptor polypeptide (GHS-R1b) is localized in the endoplasmic reticulum where it forms heterodimers with ghrelin receptors (GHS-R1a) to attenuate their cell surface expression. *Mol. Cell. Endocrinol. 348*, 247−254.
(22) Chu, K. M., Chow, K. B., Leung, P. K., Lau, P. N., Chan, C. B., Cheng, C. H., and Wise, H. (2007) Over-expression of the truncated ghrelin receptor polypeptide attenuates the constitutive activation of phosphatidylinositol-specific phospholipase C by ghrelin receptors but has no effect on ghrelin-stimulated extracellular signal-regulated kinase 1/2 activity. *Int. J. Biochem. Cell Biol. 39*, 752−764.
(23) Kern, A., Albarran-Zeckler, R., Walsh, H. E., and Smith, R. G. (2012) Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. *Neuron 73*, 317−332.
(24) Jiang, H., Betancourt, L., and Smith, R. G. (2006) Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol. Endocrinol. 20*, 1772−1785.
(25) Rediger, A., Piechowski, C. L., Yi, C. X., Tarnow, P., Strotmann, R., Gruters, A., Krude, H., Schoneberg, T., Tschop, M. H., Kleinau, G., and Biebermann, H. (2011) Mutually opposite signal modulation by hypothalamic heterodimerization of ghrelin and melanocortin-3 receptors. *J. Biol. Chem. 286*, 39623−39631.
(26) Rediger, A., Tarnow, P., Bickenbach, A., Schaefer, M., Krude, H., Grüters, A., and Biebermann, H. (2009) Heterodimerization of hypothalamic G-protein-coupled receptors involved in weight regulation. *Obes. Facts 2*, 80−86.
(27) Schellekens, H., van Oeffelen, W. E., Dinan, T. G., and Cryan, J. F. (2013) Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. *J. Biol. Chem. 288*, 181−191.
(28) Clifton, P. G., and Kennett, G. A. (2006) Monoamine receptors in the regulation of feeding behaviour and energy balance. *CNS Neurol. Disord.: Drug Targets 5*, 293−312.
(29) Halford, J. C., Harrold, J. A., Boyland, E. J., Lawton, C. L., and Blundell, J. E. (2007) Serotonergic drugs: effects on appetite expression and use for the treatment of obesity. *Drugs 67*, 27−55.
(30) Lam, D. D., Przydzial, M. J., Ridley, S. H., Yeo, G. S., Rochford, J. J., O'Rahilly, S., and Heisler, L. K. (2008) Serotonin 5-HT2C receptor agonist promotes hypophagia via downstream activation of melanocortin 4 receptors. *Endocrinology 149*, 1323−1328.
(31) Dutton, A. C., and Barnes, N. M. (2006) Anti-obesity pharmacotherapy: future perspectives utilising 5-HT2C receptor agonists. *Drug Discovery Today: Ther. Strategies 3*, 577−583.
(32) Garfield, A. S., and Heisler, L. K. (2009) Pharmacological targeting of the serotonergic system for the treatment of obesity. *J. Physiol. 587*, 49−60.
(33) Miller, K. J. (2005) Serotonin 5-HT2C receptor agonists: potential for the treatment of obesity. *Mol. Interventions 5*, 282−291.
(34) Somerville, E. M., Horwood, J. M., Lee, M. D., Kennett, G. A., and Clifton, P. G. (2007) 5-HT_{2C} receptor activation inhibits appetitive and consummatory components of feeding and increases brain c-fos immunoreactivity in mice. *Eur. J. Neurosci. 25*, 3115−3124.
(35) Tecott, L. H. (2007) Serotonin and the orchestration of energy balance. *Cell Metab. 6*, 352−361.
(36) Tecott, L. H., Sun, L. M., Akana, S. F., Strack, A. M., Lowenstein, D. H., Dallman, M. F., and Julius, D. (1995) Eating disorder and epilepsy in mice lacking 5-HT^{2C} serotonin receptors. *Nature 374*, 542−546.
(37) Vickers, S. P., Clifton, P. G., Dourish, C. T., and Tecott, L. H. (1999) Reduced satiating effect of *d*-fenfluramine in serotonin 5-HT_{2C} receptor mutant mice. *Psychopharmacology 143*, 309−314.
(38) Schellekens, H., Clarke, G., Jeffery, I. B., Dinan, T. G., and Cryan, J. F. (2012) Dynamic 5-HT2C receptor editing in a mouse model of obesity. *PLoS One 7*, e32266.
(39) Feijo, F. d. M., Bertoluci, M. C., and Reis, C. (2011) Serotonin and hypothalamic control of hunger: a review. *Rev. Assoc. Med. Bras. 57*, 74−77.
(40) Wurtman, R. J., and Wurtman, J. J. (1988) Do carbohydrates affect food intake via neurotransmitter activity? *Appetite 11*, 42−47.
(41) Dalton, G. L., Lee, M. D., Kennett, G. A., Dourish, C. T., and Clifton, P. G. (2004) mCPP-induced hyperactivity in 5-HT2C receptor mutant mice is mediated by activation of multiple 5-HT receptor subtypes. *Neuropharmacology 46*, 663−671.
(42) Dalton, G. L., Lee, M. D., Kennett, G. A., Dourish, C. T., and Clifton, P. G. (2006) Serotonin 1B and 2C receptor interactions in the modulation of feeding behaviour in the mouse. *Psychopharmacology 185*, 45−57.
(43) Lam, D. D., and Heisler, L. K. (2007) Serotonin and energy balance: molecular mechanisms and implications for type 2 diabetes. *Expert Rev. Mol. Med. 9*, 1−24.
(44) Jandacek, R. J. (2005) APD-356 (Arena). *Curr. Opin. Invest. Drugs 6*, 1051−1056.
(45) Redman, L. M., and Ravussin, E. (2010) Lorcaserin for the treatment of obesity. *Drugs Today 46*, 901−910.
(46) O'Neil, P. M., Smith, S. R., Weissman, N. J., Fidler, M. C., Sanchez, M., Zhang, J., Raether, B., Anderson, C. M., and Shanahan, W. R. (2012) Randomized placebo-controlled clinical trial of lorcaserin for weight loss in type 2 diabetes mellitus: The BLOOM-DM Study. *Obesity 7*, 1426−1436.
(47) Martin, C. K., Redman, L. M., Zhang, J., Sanchez, M., Anderson, C. M., Smith, S. R., and Ravussin, E. (2011) Lorcaserin, a 5-HT_{2C} receptor agonist, reduces body weight by decreasing energy intake without influencing energy expenditure. *J. Clin. Endocrinol. Metab. 96*, 837−845.
(48) Clemett, D. A., Punhani, T., Duxon, M. S., Blackburn, T. P., and Fone, K. C. (2000) Immunohistochemical localisation of the 5-HT_{2C} receptor protein in the rat CNS. *Neuropharmacology 39*, 123−132.
(49) Leysen, J. E. (2004) 5-HT2 receptors. *Curr. Drug Targets: CNS Neurol. Disord. 3*, 11−26.
(50) Guan, X. M., Yu, H., Palyha, O. C., McKee, K. K., Feighner, S. D., Sirinathsinghji, D. J., Smith, R. G., van der Ploeg, L. H., and Howard, A. D. (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res. 48*, 23−29.
(51) Zigman, J. M., Jones, J. E., Lee, C. E., Saper, C. B., and Elmquist, J. K. (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J. Comp. Neurol. 494*, 528−548.

DOI: 10.1021/cn500318q
ACS Chem. Neurosci. 2015, 6, 1186−1197

(52) Cowley, M. A., Smith, R. G., Diano, S., Tschop, M., Pronchuk, N., Grove, K. L., Strasburger, C. J., Bidlingmaier, M., Esterman, M., Heiman, M. L., Garcia-Segura, L. M., Nillni, E. A., Mendez, P., Low, M. J., Sotonyi, P., Friedman, J. M., Liu, H., Pinto, S., Colmers, W. F., Cone, R. D., and Horvath, T. L. (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron 37*, 649−661.

(53) Brunetti, L., Recinella, L., Orlando, G., Michelotto, B., Di Nisio, C., and Vacca, M. (2002) Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. *Eur. J. Pharmacol. 454*, 189−192.

(54) Ghersi, M. S., Casas, S. M., Escudero, C., Carlini, V. P., Buteler, F., Cabrera, R. J., Schioth, H. B., and de Barioglio, S. R. (2011) Ghrelin inhibited serotonin release from hippocampal slices. *Peptides 32*, 2367−2371.

(55) Hansson, C., Alvarez-Crespo, M., Taube, M., Skibicka, K. P., Schmidt, L., Karlsson-Lindahl, L., Egecioglu, E., Nissbrandt, H., and Dickson, S. L. (2014) Influence of ghrelin on the central serotonergic signaling system in mice. *Neuropharmacology 79*, 498−505.

(56) Nonogaki, K., Ohashi-Nozue, K., and Oka, Y. (2006) A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice. *Biochem. Biophys. Res. Commun. 341*, 703−707.

(57) Currie, P. J., John, C. S., Nicholson, M. L., Chapman, C. D., and Loera, K. E. (2010) Hypothalamic paraventricular 5-hydroxytryptamine inhibits the effects of ghrelin on eating and energy substrate utilization. *Pharmacol., Biochem. Behav. 97*, 152−155.

(58) Cabral, A., Fernandez, G., and Perello, M. (2013) Analysis of brain nuclei accessible to ghrelin present in the cerebrospinal fluid. *Neuroscience 253*, 406−415.

(59) Cabral, A., Valdivia, S., Fernandez, G., Reynaldo, M., and Perello, M. (2014) Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility. *J. Neuroendocrinol. 26*, 542−554.

(60) Berg, K. A., Clarke, W. P., Cunningham, K. A., and Spampinato, U. (2008) Fine-tuning serotonin2c receptor function in the brain: molecular and functional implications. *Neuropharmacology 55*, 969−976.

(61) Berg, K. A., Cropper, J. D., Niswender, C. M., Sanders-Bush, E., Emeson, R. B., and Clarke, W. P. (2001) RNA-editing of the 5-HT_{2C} receptor alters agonist-receptor-effector coupling specificity. *Br. J. Pharmacol. 134*, 386−392.

(62) Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E., and Emeson, R. B. (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature 387*, 303−308.

(63) Herrick-Davis, K., Grinde, E., and Niswender, C. M. (1999) Serotonin 5-HT_{2C} receptor RNA editing alters receptor basal activity: implications for serotonergic signal transduction. *J. Neurochem. 73*, 1711−1717.

(64) Wang, Q., O'Brien, P. J., Chen, C. X., Cho, D. S., Murray, J. M., and Nishikura, K. (2000) Altered G protein-coupling functions of RNA editing isoform and splicing variant serotonin2C receptors. *J. Neurochem. 74*, 1290−1300.

(65) Niswender, C. M., Copeland, S. C., Herrick-Davis, K., Emeson, R. B., and Sanders-Bush, E. (1999) RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. *J. Biol. Chem. 274*, 9472−9478.

(66) Olaghere da Silva, U. B., Morabito, M. V., Canal, C. E., Airey, D. C., Emeson, R. B., and Sanders-Bush, E. (2010) Impact of RNA editing on functions of the serotonin 2C receptor in vivo. *Front. Neurosci. 4*, 26.

(67) Werry, T. D., Loiacono, R., Sexton, P. M., and Christopoulos, A. (2008) RNA editing of the serotonin 5HT2C receptor and its effects on cell signalling, pharmacology and brain function. *Pharmacol. Ther. 119*, 7−23.

(68) Kawahara, Y., Grimberg, A., Teegarden, S., Mombereau, C., Liu, S., Bale, T. L., Blendy, J. A., and Nishikura, K. (2008) Dysregulated editing of serotonin 2C receptor mRNAs results in energy dissipation and loss of fat mass. *J. Neurosci. 28*, 12834−12844.

(69) Kohno, D., Gao, H. Z., Muroya, S., Kikuyama, S., and Yada, T. (2003) Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes 52*, 948−956.

(70) Suzuki, K., Simpson, K. A., Minnion, J. S., Shillito, J. C., and Bloom, S. R. (2010) The role of gut hormones and the hypothalamus in appetite regulation. *Endocr. J. 57*, 359−372.

(71) Simpson, K. A., Martin, N. M., and Bloom, S. R. (2009) Hypothalamic regulation of food intake and clinical therapeutic applications. *Arq. Bras. Endocrinol. Metabol. 53*, 120−128.

(72) Kanoski, S. E., Hayes, M. R., Greenwald, H. S., Fortin, S. M., Gianessi, C. A., Gilbert, J. R., and Grill, H. J. (2011) Hippocampal leptin signaling reduces food intake and modulates food-related memory processing. *Neuropsychopharmacology 36*, 1859−1870.

(73) Bubar, M. J., and Cunningham, K. A. (2007) Distribution of serotonin 5-HT_{2C} receptors in the ventral tegmental area. *Neuroscience 146*, 286−297.

(74) Pompeiano, M., Palacios, J. M., and Mengod, G. (1994) Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A and 5-HT2C receptors. *Brain Res. Mol. Brain Res. 23*, 163−178.

(75) Bonn, M., Schmitt, A., and Asan, E. (2012) Double and triple in situ hybridization for coexpression studies: combined fluorescent and chromogenic detection of neuropeptide Y (NPY) and serotonin receptor subtype mRNAs expressed at different abundance levels. *Histochem. Cell Biol. 137*, 11−24.

(76) Bonn, M., Schmitt, A., Lesch, K. P., Van Bockstaele, E. J., and Asan, E. (2013) Serotonergic innervation and serotonin receptor expression of NPY-producing neurons in the rat lateral and basolateral amygdaloid nuclei. *Brain Struct. Funct. 218*, 421−435.

(77) Diano, S. (2008) Ghrelin's role in feeding behavior and memory performance. *Appetite 51*, 363−363.

(78) Diano, S., Farr, S. A., Benoit, S. C., McNay, E. C., da Silva, I., Horvath, B., Gaskin, F. S., Nonaka, N., Jaeger, L. B., Banks, W. A., Morley, J. E., Pinto, S., Sherwin, R. S., Xu, L., Yamada, K. A., Sleeman, M. W., Tschop, M. H., and Horvath, T. L. (2006) Ghrelin controls hippocampal spine synapse density and memory performance. *Nat. Neurosci. 9*, 381−388.

(79) Schwartz, G. J. (2000) The role of gastrointestinal vagal afferents in the control of food intake: current prospects. *Nutrition 16*, 866−873.

(80) Benoit, S., Schwartz, M., Baskin, D., Woods, S. C., and Seeley, R. J. (2000) CNS melanocortin system involvement in the regulation of food intake. *Horm. Behav. 37*, 299−305.

(81) Branchek, T. A., and Gershon, M. D. (1989) Time course of expression of neuropeptide Y, calcitonin gene-related peptide, and NADPH diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxytryptamine in mucosal enterochromaffin cells. *J. Comp. Neurol. 285*, 262−273.

(82) McGirr, R., McFarland, M. S., McTavish, J., Luyt, L. G., and Dhanvantari, S. (2011) Design and characterization of a fluorescent ghrelin analog for imaging the growth hormone secretagogue receptor 1a. *Regul. Pept. 172*, 69−76.

(83) Finger, B. C., Schellekens, H., Dinan, T. G., and Cryan, J. F. (2011) Is there altered sensitivity to ghrelin-receptor ligands in leptin-deficient mice?: importance of satiety state and time of day. *Psychopharmacology 216*, 421−429.

(84) Diz-Chaves, Y. (2011) Ghrelin, appetite regulation, and food reward: interaction with chronic stress. *Int. J. Pept. 2011*, 898450.

(85) Chuang, J. C., Perello, M., Sakata, I., Osborne-Lawrence, S., Savitt, J. M., Lutter, M., and Zigman, J. M. (2011) Ghrelin mediates stress-induced food-reward behavior in mice. *J. Clin. Invest. 121*, 2684−2692.

(86) Schellekens, H., Dinan, T. G., and Cryan, J. F. (2013) Ghrelin at the interface of obesity and reward. *Vitam. Horm. 91*, 285−323.

DOI: 10.1021/cn500318q
ACS Chem. Neurosci. 2015, 6, 1186−1197

(87) Schellekens, H., Finger, B. C., Dinan, T. G., and Cryan, J. F. (2012) Ghrelin signalling and obesity: at the interface of stress, mood and food reward. *Pharmacol. Ther. 135*, 316−326.
(88) Skibicka, K. P., Hansson, C., Egecioglu, E., and Dickson, S. L. (2012) Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and acetylcholine receptor gene expression. *Addict. Biol. 17*, 95−107.
(89) Skibicka, K. P., and Dickson, S. L. (2011) Ghrelin and food reward: the story of potential underlying substrates. *Peptides 32*, 2265−2273.
(90) Egecioglu, E., Skibicka, K. P., Hansson, C., Alvarez-Crespo, M., Friberg, P. A., Jerlhag, E., Engel, J. A., and Dickson, S. L. (2011) Hedonic and incentive signals for body weight control. *Rev. Endocr. Metab. Disord. 12*, 141−151.
(91) Dickson, S. L., Egecioglu, E., Landgren, S., Skibicka, K. P., Engel, J. A., and Jerlhag, E. (2011) The role of the central ghrelin system in reward from food and chemical drugs. *Mol. Cell. Endocrinol. 340*, 80−87.
(92) Perello, M., and Zigman, J. M. (2012) The role of ghrelin in reward-based eating. *Biol. Psychiatry 72*, 347−353.
(93) Alex, K. D., and Pehek, E. A. (2007) Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission. *Pharmacol. Ther. 113*, 296−320.
(94) Higgins, G. A., and Fletcher, P. J. (2003) Serotonin and drug reward: focus on 5-HT_{2C} receptors. *Eur. J. Pharmacol. 480*, 151−162.
(95) Liu, S., and Borgland, S. L. (2015) Regulation of the mesolimbic dopamine circuit by feeding peptides. *Neuroscience 289C*, 19−42.
(96) Di Matteo, V., Cacchio, M., Di Giulio, C., and Esposito, E. (2002) Role of serotonin(2C) receptors in the control of brain dopaminergic function. *Pharmacol., Biochem. Behav. 71*, 727−734.
(97) Skibicka, K. P., Hansson, C., Alvarez-Crespo, M., Friberg, P. A., and Dickson, S. L. (2011) Ghrelin directly targets the ventral tegmental area to increase food motivation. *Neuroscience 180*, 129−137.
(98) Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J., and Baskin, D. G. (2000) Central nervous system control of food intake. *Nature 404*, 661−671.
(99) Schellekens, H., McNamara, O., Dinan, T. G., McCarthy, J. V., McGlacken, G. P., and Cryan, J. F. (2013) Semagacestat, a gamma-secretase inhibitor, activates the growth hormone secretagogue (GHS-R1a) receptor. *J. Pharm. Pharmacol. 65*, 528−538.
(100) Morabito, M. V., Abbas, A. I., Hood, J. L., Kesterson, R. A., Jacobs, M. M., Kump, D. S., Hachey, D. L., Roth, B. L., and Emeson, R. B. (2010) Mice with altered serotonin 2C receptor RNA editing display characteristics of Prader−Willi syndrome. *Neurobiol Dis 39*, 169−180.
(101) Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods 9*, 676−682.
(102) Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Ueno, N., Makino, S., Fujimiya, M., Niijima, A., Fujino, M. A., and Kasuga, M. (2001) Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology 120*, 337−345.

DOI: 10.1021/cn500318q
ACS Chem. Neurosci. 2015, 6, 1186−1197