Ga1o-coupled Htr2c in the paraventricular nucleus of the hypothalamus antagonizes the anorectic effect of serotonin agents

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SUMMARY

The anorexigenic effect of serotonergic compounds has largely been attributed to activation of serotonin 2C receptors (Htr2cs). Using mouse genetic models in which Htr2c can be selectively deleted or restored (in Htr2c-null mice), we investigate the role of Htr2c in forebrain Sim1
neurons. Unexpectedly, we find that *Htr2c* acts in these neurons to promote food intake and counteract the anorectic effect of serotonergic appetite suppressants. Furthermore, *Htr2c* marks a subset of *Sim1* neurons in the paraventricular nucleus of the hypothalamus (PVH). Chemogenetic activation of these neurons in adult mice suppresses hunger, whereas their silencing promotes feeding. In support of an orexigenic role of PVH *Htr2c*, whole-cell patch-clamp experiments demonstrate that activation of *Htr2c* inhibits PVH neurons. Intriguingly, this inhibition is due to Ga\textsubscript{i/o}-dependent activation of ATP-sensitive K\textsuperscript{+} conductance, a mechanism of action not identified previously in the mammalian nervous system.

**Graphical Abstract**

![Graphical Abstract](image)

**In brief**

Yoo et al. show that *Htr2c*, the target of a former weight loss drug, can inhibit and promote food intake by coupling with distinct intracellular signaling events in different hypothalamic neurons. These findings may help explain the rather modest anti-obesity effects of *Htr2c* agonists.

**INTRODUCTION**

To combat the obesity epidemic as well as eating disorders such as anorexia nervosa, it is essential that we gain a better understanding of the complex regulation of feeding behaviors. Recent studies have identified several “feeding” neurons along the neuraxis where hunger and satiety cues are integrated with cognitive, emotional, and reward inputs (Kenny, 2011;
Schwartz and Zeltser, 2013). These neuron populations may adopt different strategies to integrate neuronal and humoral signals and exert a positive or negative influence on food intake. For example, it has been shown that agouti-related protein (AgRP) neurons evoke voracious feeding behaviors through parallel and redundant forebrain feeding circuits, each of which is sufficient to drive food intake (Betley et al., 2013). On the other hand, multiple anorexigenic signals converge onto protein kinase C (PKC)-δ+ neurons in the amygdala, supporting a model where diverse anorectic information is processed in one central node (Cai et al., 2014).

The brain serotonin (5-hydroxytryptamine or 5-HT) system is a key target for weight loss therapies. Pharmacological agents that increase extracellular 5-HT content suppress food intake and reduce body weight (Wyler et al., 2017). The therapeutic potential of this pathway was highlighted by the demonstration that the anorexigenic actions of serotoninergic agents such as d-fenfluramine (dFen, a key ingredient of the once-popular diet pill Fen/Phen) were mediated in part through the serotonin 2c receptor (Htr2c) (Tecott et al., 1995; Vickers et al., 1999). Subsequently, lorcaserin, an Htr2c-specific agonist, became one of the first US Food and Drug Administration (FDA)-approved weight loss drugs (Colman et al., 2012).

Htr2c is widely distributed in the brain, including in regions that are known to regulate food intake (Hoffman and Mezey, 1989). However, it is not clear whether the anorectic effects of 5-HT agents require coordinated actions of Htr2c at multiple feeding nodes or whether activation of Htr2c in discrete neuronal populations suppresses food intake in a parallel or convergent manner. We previously demonstrated that selective restoration of Htr2c only in pro-opiomelanocortin (POMC) neurons of Htr2c-null mice was sufficient to normalize body weight, food intake, and the anorectic responses to dFen (Xu et al., 2008). Conversely, deletion of Htr2c specifically in these neurons during development or in adulthood resulted in hyperphagia and a blunted anorectic response to dFen (Berglund et al., 2013; D’Agostino et al., 2018). These findings identified POMC neurons as one of the sites where Htr2c regulates food intake.

In addition to POMC neurons, Htr2c is enriched in neurons of the paraventricular nucleus of the hypothalamus (PVH) that express the transcription factor single-minded 1 (Sim1) (Heisler et al., 2007). PVH Sim1 neurons are physiologically relevant to regulation of feeding behaviors because haploinsufficiency of Sim1 results in reduced numbers of PVH neurons and causes early-onset hyperphagia and obesity in rodents and humans (Holder et al., 2004; Michaud et al., 2001). Furthermore, deletion of methyl CpG binding protein 2 (Fyffe et al., 2008) or melanocortin 4 receptor (MC4R) specifically in Sim1 neurons leads to hyperphagia and obesity in rodents (Li et al., 2021). Importantly, we demonstrated that the anorectic responses to 5-HT agents were blunted in mice heterozygous for Sim1, suggesting that Sim1 neurons represent another important node in the brain to regulate such responses (Xu et al., 2010). In the current study, we investigated the role of Htr2c in feeding regulation in Sim1 neurons using a combination of mouse genetic, chemogenetic, and electrophysiological analyses. Our studies led to the unexpected finding that Htr2c acts in Sim1 neurons to promote food intake and counteract the anorexigenic actions of 5-HT agents. Moreover, although activation of Htr2c depolarizes anorexigenic POMC neurons in
a phospholipase C (PLC)-dependent manner (Sohn et al., 2011), we find that its actions on PVH neurons are via the Go/i/o pathway, resulting in hyperpolarization of PVH neurons.

RESULTS

Selective restoration of Htr2c only in Sim1 neurons promotes food intake

We previously generated and characterized a re-activatable Htr2c-null mouse (Htr2c^{null}) in which transcription of endogenous Htr2c was impeded by a transcriptional blocker cassette. Specific to this model, physiological levels of Htr2c can be restored selectively in distinct cell groups upon Cre recombinase activity. Consistent with previous reports (Nonogaki et al., 1998; Xu et al., 2008), chow-fed Htr2c^{null} mice were hyperphagic in a fasting-refeeding test but had body weight gains comparable with their wild-type littermates until 20 weeks of age because of a concomitant increase in energy expenditure (Figure S1A). However, hyperphagia was exacerbated when these mice were fed a high-fat diet (HFD; 42% kcal from fat, 0.2% cholesterol), tipping the scale of energy balance. As a result, despite the sustained higher energy expenditure, male Htr2c^{null} mice rapidly gained more weight and became significantly heavier than their littermate controls after an average of 8–10 weeks of HFD feeding (Figure 1A).

To examine whether selective restoration of Htr2c in Sim1 neurons was sufficient to normalize the body weight deficits of Htr2c^{null} mice, we bred re-activatable Htr2c^{null} mice with Sim1::Cre mice (Balthasar et al., 2005) to generate Htr2c^{null}, Sim1::Cre mice in which endogenous levels of Htr2c were restored only in Sim1 neurons (hereafter called Htr2c^{Sim1RE} mice). We found that selective re-expression of Htr2c in Sim1 neurons failed to normalize hyperphagia or higher energy expenditure in chow-fed mice (Figures S1B-S1D). In contrast, HFD-fed Htr2c^{Sim1RE} mice gained significantly more weight and became obese faster than their wild-type (Sim1::Cre +/−) and Htr2c^{null} littermates (Figure 1A). We then subjected another cohort of mice to a combined indirect calorimetry system (TSE metabolic chambers) where food intake, energy expenditure, and physical activity were monitored continuously. We found that, although the abnormal increase in energy expenditure persisted and remained comparable between HFD-fed Htr2c^{null} and Htr2c^{Sim1RE} mice (Figure 1B), hyperphagia was exaggerated in Htr2c^{Sim1RE} mice because they consumed significantly more calories than Htr2c^{null} mice during the dark phase, when mice are normally active (Figure 1C). The respiratory exchange ratio was not different among Htr2c^{Sim1RE}, Htr2c^{null}, and wild-type mice (Figure 1D).

Next we investigated whether Htr2c in Sim1 neurons regulated the anorexigenic effects of serotonergic compounds such as dFen, a 5-HT releaser and uptake inhibitor, and meta-chlorophenylpiperazine (mCPP), a non-selective Htr2c agonist. Refeeding was measured in chow-fed mice after an overnight fast followed by an intraperitoneal (i.p.) dose of drug or saline. Despite the sustained effect on cumulative intake, the anorexigenic actions of dFen (6 mg/kg) and mCPP (5 mg/kg) were most prominent within the first 2 h following drug administration, whereas refeeding during subsequent hours was not statistically different between mice treated with drug or saline (Figures S1E-S1H). It has been reported previously that the anorexigenic effects of dFen and mCPP are blunted in conventional Htr2c^{null} mice (Tecott et al., 1995; Vickers et al., 1999). Similarly, we found that Htr2c^{null}
mice showed attenuated anorectic responses to these agents (Xu et al., 2008). However, we unexpectedly found that such responses were blunted further in Htr2c<sup>Sim1RE</sup> mice (Figures 1E and 1F). We then tested the response to lorcaserin, a selective agonist for Htr2c (Thomsen et al., 2008). As expected, lorcaserin (10 mg/kg) inhibited food intake in wild-type littermates but not in Htr2c<sup>null</sup> mice (Figure 1G). Surprisingly, rather than suppressing food intake, lorcaserin appeared to increase food intake in Htr2c<sup>Sim1RE</sup> mice (Figure 1G).

**Selective ablation of Htr2c in Sim1 neurons reduces food intake**

Results from the re-expression studies raised the intriguing possibility that, despite its anorexigenic function in hypothalamic POMC neurons, Htr2c may act in Sim1 neurons to promote food intake. To test this hypothesis, we selectively deleted Htr2c in Sim1 neurons by breeding floxed Htr2c mice (Berglund et al., 2013) with Sim1::Cre mice to generate Htr2c<sup>f/f</sup>; Sim1::Cre mice (hereafter called as Htr2c<sup>Sim1KO</sup>). Sim1::Cre activity is enriched in neurons in the PVH and amygdala (Balthasar et al., 2005). In situ hybridization analyses demonstrated that Htr2c mRNAs were downregulated significantly in the PVH of Htr2c<sup>Sim1KO</sup> mice, whereas levels within the amygdala were comparable between the two genotypes (Figures 2A-2D). Compared with their littermate controls (Htr2c<sup>f/f</sup>), Htr2c<sup>Sim1KO</sup> mice had normal body weight and body composition at 6 weeks of age (Figures 2E and 2F). Moreover, these mice demonstrated normal glucoregulatory responses in glucose and insulin tolerance tests (Figures S2A and S2B). However, Htr2c<sup>Sim1KO</sup> mice gained less weight and became significantly leaner than their littermate controls when fed an HFD (Figure 2E). Nuclear magnetic resonance analyses revealed that the decreased body weight in Htr2c<sup>Sim1KO</sup> mice was due to a reduction in fat and lean mass (Figure 2F).

Furthermore, metabolic chamber analyses of a separate cohort of mice showed that food intake was reduced in HFD-fed Htr2c<sup>Sim1KO</sup> mice (Figure 2G), whereas energy expenditure (Figure 2H) and physical activity (Figure 2I) remained comparable with their littermate controls. Interestingly, we found that chow-fed Htr2c<sup>Sim1KO</sup> mice exhibited an anorectic response to a sub-anorectic dose of dFen (1 mg/kg) that otherwise did not suppress refeeding (during the first 2 h) in wild-type littermates (Figure 2J; Wurtman and Wurtman, 1977). Likewise, the anorectic response to mCPP (5 mg/kg) was also potentiated in these mice (Figure 2K). These findings suggest that Htr2c in Sim1 neurons modulates the anorectic responses to 5-HT compounds.

PVH neurons are critical regulators of stress responses (Xu et al., 2019). Therefore, we investigated whether the observed decrease in food intake in Htr2c<sup>Sim1KO</sup> mice was due to distress, such as increased anxiogenic and/or depressive behaviors. We tested two independent cohorts of control and Htr2c<sup>Sim1KO</sup> mice that were fed chow (data not shown) or an HFD in a series of behavioral paradigms including the elevated plus maze, dark-light exploration, open field tests for anxiety-related behaviors, as well as the forced swim test for depression-like behaviors (Figures S2C-S2F). However, we found no differences between the genotypes in any of these tests, indicating that the reduced food intake was unlikely to be the result of mood disturbances.
**PVH *Htr2c* neurons bidirectionally regulate food intake**

To investigate the neurochemical identity of PVH *Htr2c* neurons, we conducted Cre-mediated fate mapping using a *Htr2c::Cre* mouse we developed recently, in which expression of a Cre recombinase is driven by the endogenous regulatory sequences of *Htr2c* (Park et al., 2020). We found that expression of a Cre-activated tdTomato reporter (Ai14) marked a subset of adult PVH neurons. None of these neurons expressed PVH neuronal markers such as oxytocin (OXY; Figure S3), arginine vasopressin (AVP), or tyrosine hydroxylase (TH). PVH neurons expressing *Mc4r* mediate the anorectic effect of serotonin downstream of *Htr2c/POMC* neurons (Xu et al., 2010). However, none of the tomato-positive neurons in the PVH expressed *Mc4r-GFP*. Using double immunohistochemistry and *in situ* hybridization (Figure S3), we found that the majority of PVH *Htr2c* neurons expressed mRNAs for corticotropin-releasing hormone (*Crh*) or thyrotropin-releasing hormone (*Trh*). Furthermore, similar analyses showed that these neurons did not express pituitary adenylate cyclase-activating polypeptide (*Pacap*) or galanin (*Gal*).

To test whether PVH *Htr2c* neurons directly regulate food intake *in vivo*, we stereotaxically delivered adeno-associated viruses (AAVs) expressing Cre-dependent Designer Receptors Exclusively Activated by Designer Drugs (DREADD) constructs (Krashes et al., 2011) into the PVH of *Htr2c::Cre* mice (Figures 3A and 3B). Following an i.p. dose of clozapine-N-oxide (CNO; 1 mg/kg), we found elevated levels of Fos protein, a marker for enhanced neuronal activity, on the ipsilateral side of the PVH that received the stimulatory DREADD (hM3Dq) (Figure 3C; 95.89 ± 6.18 positive cells/section compared with 14.56 ± 4.02 positive cells/section on the contralateral side). Fos expression was found in the majority (86.84% ± 4.25% of 469 cells from three mice) of *Htr2c* neurons that had been targeted by the GFP-labeled hM3Dq (Figure 3D). Consistent with this, electrophysiological recordings of GFP-labeled neurons showed that bath application of CNO (5 μM) elicited rapid depolarization of membrane potentials in PVH *Htr2c* neurons (Figures 3E and 3F). In *Htr2c::Cre* mice that received unilateral or bilateral hM3Dq injections in the PVH, a dose of CNO (i.p., 1 mg/kg) suppressed fasting-induced refeeding (Figure 3G). Importantly, CNO had no effect on food intake in mice in which the stereotactic injections were placed outside of the PVH (misinjection; Figure 3G), nor did it alter feeding in *Htr2c::Cre* mice that received injections of a control virus (AAV-DIO-mCherry; Figure S4). Therefore, these findings demonstrate that chemogenetic activation of PVH *Htr2c* neurons inhibits feeding.

To evaluate the effect of silencing PVH *Htr2c* neurons on food intake, we conducted bilateral stereotaxic injections of AAVs that contained the inhibitory DREADD (hM4Di). Overnight-fasted mice were initially satiated for 1 h before receiving either CNO or saline. We found that individual mice consumed similar amounts of food during the satiation period (Figure 3H). However, compared with saline-treated mice, silencing PVH *Htr2c* neurons increased food intake after CNO treatment (Figure 3I). These findings demonstrated that PVH *Htr2c* neurons can bidirectionally regulate food intake *in vivo*. The effects of chemogenetic manipulations (stimulation or silencing) on food intake were transient because there were no differences in cumulative food intake between saline- and CNO-treated mice after 24 h (Figure S4).
**Htr2c agonists hyperpolarize PVH neurons via postsynaptic K\textsubscript{ATP} channels**

Our chemogenetic experiments demonstrated that activation of PVH Htr2c neurons produced an anorectic effect. In contrast, our genetic studies appeared to suggest that Htr2c acts in PVH neurons to promote feeding. Htr2c has been reported to couple to the G\textsubscript{α}q/PLC pathway and, upon activation, to depolarize membrane potentials (Sohn et al., 2011). How could activation of Htr2c in anorexigenic PVH Htr2c neurons ultimately lead to an orexigenic effect? To investigate this apparent discrepancy, we studied the acute effects of Htr2c agonists on membrane potentials of genetically labeled Htr2c neurons in the arcuate nucleus of the hypothalamus (ARH) and the PVH.

We have demonstrated that activation of Htr2c depolarized a subset of ARH POMC neurons (Sohn et al., 2011). Consistent with this finding, mCPP (4 μM) depolarized a subpopulation of tomato-positive Htr2c neurons within the ARH (Figure S5). However, to our surprise, mCPP hyperpolarized the membrane potential of Htr2c neurons in the PVH by −9.9 ± 0.8 mV (28 of 76 cells, 36.8%; Figure 4A). The mCPP-induced hyperpolarization was not blunted by the presence of tetrodotoxin (TTX; 0.5 μM) and a cocktail of fast synaptic blockers (10 μM CNQX + 50 μM AP5 + 50 μM picrotoxin), supporting a postsynaptic event (−7.8 ± 0.9 mV, 6 of 11 cells, 54.5%; Figure 4B). We further tested the acute responses to the Htr2c-specific agonist lorcaserin and found that lorcaserin hyperpolarized PVH Htr2c neurons by −10.2 ± 2.2 mV (5 of 11 cells, 45.5%; Figure 4C). No depolarizing responses were recorded in the presence of mCPP (n = 76) or lorcaserin (n = 11). Moreover, lorcaserin-induced hyperpolarization of PVH Htr2c neurons was accompanied by an 18.6% ± 4.6% decrease in input resistance (from 1,074 ± 109 MΩ to 875 ± 103 MΩ, n = 5), which was comparable with that induced by mCPP treatment (decreased 20.5% ± 1.7% from 1,335 ± 53 MΩ to 1,056 ± 103 MΩ, n = 28). In both cases, the reversal potential (E\textsubscript{rev}; −94.7 ± 7.1 mV for lorcaserin, −97.0 ± 2.4 mV for mCPP) was indicative of activated potassium conductance (Figure 4D). Consistent with these findings, we found that the mCPP-induced hyperpolarization in PVH Htr2c neurons was completely reversed by the ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channel inhibitors tolbutamide (200 μM; Figures 4A and 4E) and glibenclamide (100 μM; Figure 4F). Similarly, 5-HT (30 μM) hyperpolarized PVH Htr2c neurons (−9.2 ± 1.4 mV, 6 of 12 cells, 50.0%; Figures 4G and 4H), presumably by increasing potassium conductance (22.8% ± 1.4% decrease in input resistance from 1,571 ± 74 MΩ to 1,216 ± 71 MΩ, E\textsubscript{rev} = −86.4 ± 4.5 mV, n = 6). Interestingly, we found that inhibition of G\textsubscript{α}q proteins/PLC signaling with anti-G\textsubscript{α}q antibodies or U73122 (a PLC inhibitor, 5 μM) failed to prevent mCPP-induced inhibition (Figures 4G and 4H, 11 of 26 cells [42.3%] pretreated with anti-G\textsubscript{α}q antibodies hyperpolarized by −7.7 ± 1.4 mV; 4 of 13 cells [30.8%] pretreated with U73122 hyperpolarized by −6.0 ± 1.1 mV, respectively). It has been reported that K\textsubscript{ATP} channels can couple to the G\textsubscript{α}i/o pathway to hyperpolarize membrane potentials (Kurachi et al., 1992). In support of this, we found that application of pertussis toxin (PTX; 1 ng/μL), which inhibits G\textsubscript{α}i/o proteins, prevented mCPP-induced hyperpolarization of PVH Htr2c neurons (Figures 4G and 4H). These findings suggest that Htr2c couples to G\textsubscript{α}i/o-K\textsubscript{ATP} pathway in PVH neurons to inhibit their activity and promote feeding.
DISCUSSION

Despite its pharmacological effect on appetite suppression, serotonergic regulation of feeding is far more complex at the physiological level. For example, dietary depletion of tryptophan, a precursor of 5-HT biosynthesis, had little effect on food intake in healthy humans (Pagoto et al., 2009). Furthermore, mice completely lacking 5-HT in the brain or the whole body from early development are viable, fertile, and exhibit normal food intake and body weight (Mosienko et al., 2015). It has been shown that 5-HT can promote or inhibit food intake via different 5-HT receptors. For instance, its anorexigenic effect is largely attributed to activation of Htr2c and Htr1b (Lucas et al., 1998; Nonogaki et al., 1998). In contrast, treatment with 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), an Htr1a agonist, increases feeding in C57BL/6 mice (Ebenezer and Surujbally, 2007).

Employing mouse genetic models in which endogenous Htr2c is selectively ablated or restored in Sim1 neurons, our studies demonstrate that activation of Htr2c can produce orexigenic and anorexigenic effects. Similar to many other genes that are important for feeding regulation, Htr2c has broad distribution throughout the brain. Conventional wisdom has it that individual feeding regulators play coherent roles by promoting or inhibiting food intake at discrete central feeding nodes. Supporting this idea, the anorexigenic hormone leptin suppresses food intake by activating anorexigenic POMC neurons and inhibiting orexigenic AgRP neurons (Baver et al., 2014; Cowley et al., 2001; Hill et al., 2008). However, our study reveals that activation of Htr2c at different brain sites can have opposing effects on food intake. Contrary to its anorexigenic role in POMC neurons, Htr2c acts in Sim1 neurons to promote food intake. This is further supported by our findings that restoration of Htr2c only in Sim1 neurons exacerbates hyperphagia in Htr2c\textsuperscript{null} mice, whereas its selective deletion in the same neurons reduces food intake. Along this line, we show that the pharmacological effects of 5-HT agents can be differentially modulated by Htr2c in different brain locations. Opposite to its effect in POMC neurons, we find that Htr2c acts in Sim1 neurons to dampen anorectic responses. This is corroborated by the observation that lorcaserin, an Htr2c-specific agonist, suppressed feeding in wild-type animals but appeared to increase food intake in mice in which Htr2c was expressed exclusively in Sim1 neurons. We hypothesize that Htr2c in Sim1 neurons may provide important regulatory feedback that restricts the effectiveness and safety of serotonergic appetite suppressants. Moreover, these data may be a cellular correlate to the rather modest anti-obesity effects of lorcaserin that have been observed in humans (Tchang et al., 2020).

Sim1::Cre activity has been reported only in a handful of brain structures, including most prominently the PVH and medial amygdala (Balthasar et al., 2005). Our observation that Htr2c expression was largely preserved in the amygdala of Htr2c\textsuperscript{Sim1KO} mice suggests minimal overlap between Sim1::Cre and Htr2c expression in the amygdala and that the reduced food intake in these mice was not likely to be due to loss of amygdala Htr2c. These findings implicate the PVH as another critical brain site where Htr2c regulates food intake. Importantly, reactivation or deletion of Htr2c in Sim1 neurons had no effect on other physiological processes, such as energy expenditure, physical activity, or glucose homeostasis. Furthermore, mice lacking Htr2c in Sim1 neurons had normal responses in a
battery of anxiety- and depression-related behavioral tests. Our findings suggest a dedicated role of Htr2c in Sim1 neurons for feeding regulation.

Delineation of PVH feeding circuits is a challenge because the PVH consists of several functionally heterogeneous neuronal populations that differ in morphology, connectivity, and peptidergic identity (Biag et al., 2012; Swanson and Sawchenko, 1983). Our immunohistochemical analyses demonstrated that Htr2c marks a distinct population of PVH neurons that largely consist of subsets of parvocellular CRH and TRH neurons. These findings are in agreement with earlier histological data showing that 5-HT terminals in the PVH are enriched in the parvocellular division of the nucleus (Sawchenko et al., 1983). Likewise, numerous studies have established an inhibitory role of PVH neurons in food intake. With few exceptions (Krashes et al., 2014), activation of PVH neurons typically leads to suppression of food intake, whereas silencing or genetic ablation of these neurons causes hyperphagia (An et al., 2020; Atasoy et al., 2012; Li et al., 2019; Liu et al., 2017; Pei et al., 2014; Xi et al., 2013). Consistent with the anorexigenic effect of CRH and TRH (Krahn et al., 1988; Vijayan and McCann, 1977), we found that adult chemogenetic stimulation of PVH Htr2c neurons suppressed hunger-induced refeeding, whereas silencing of these neurons boosted consumption in satiated mice. These findings establish that PVH Htr2c neurons can bidirectionally regulate food intake in vivo. However, future studies are warranted to map the serotonergic inputs and downstream targets of these neurons. We have shown previously that PVH MC4Rs act downstream of the ARH Htr2c/POMC neurons to mediate the anorectic effect of 5-HT agents (Xu et al., 2010). Interestingly, PVH Htr2c neurons do not express MC4R-GFP. Furthermore, it has been shown that different hypothalamic nuclei are innervated by discrete serotonergic cell groups in the midbrain (Sawchenko et al., 1983). Therefore, we predict that Htr2c neurons in the ARH and PVH may receive inputs from discrete 5-HT neurons and activate under different physiological conditions.

Htr2c regulates food intake through homeostatic and non-homeostatic mechanisms. For example, dopamine (DA) neurons in the ventral tegmental area (VTA) express Htr2c and are activated by its specific agonist, lorcaserin (Xu et al., 2017). Given the importance of DA neurons in reward processing, Htr2c may regulate hedonic feeding in part through VTA DA neurons. In support of this notion, loss of Htr2c in these neurons attenuates lorcaserin’s inhibitory effect on binge-like eating but does not alter its anorectic effect on hunger-driven feeding (Xu et al., 2017). In the current study, we found that chow-fed Htr2cnull and Htr2cSim1KO mice were hyperphagic but had comparable food intake in a fasting-refeeding paradigm. In contrast, Htr2cSim1RE mice consumed more food than Htr2cnull mice when both were fed an HFD. Consistent with this finding, HFD-fed Htr2cSim1KO mice ate less than controls and were protected from HFD-induced weight gain. These findings support a role of Htr2c in Sim1 neurons in HFD-induced feeding adaptations. Many PVH Htr2c neurons express CRH, and a recent study has reported that reward consumption of sucrose solution inhibits PVH CRH neurons in freely behaving mice (Yuan et al., 2019). Although we showed that chemogenetic inhibition of PVH Htr2c neurons was sufficient to increase food intake in mice, future studies are warranted to determine whether HFD consumption has a similar inhibitory effect on PVH CRH neurons and whether it induces hyperphagia by inhibiting PVH CRH/Htr2c neurons.
Htr2c has been known to couple to the G\textsubscript{αq} pathway and, upon activation, is expected to increase neuronal activity. In support of this, we demonstrated previously that Htr2c agonists depolarized ARH POMC neurons in a G\textsubscript{αq}/PLC-dependent manner (Sohn et al., 2011). In the current study, we verified this finding and found that mCPP depolarized genetically labeled Htr2c/POMC neurons in the ARH of triple-transgenic Htr2c::Cre, R26R::tdTomato, Pomc::GFP mice. However, recordings in the same mice revealed that none of the PVH Htr2c neurons showed a depolarizing response to Htr2c agonist treatment. In contrast, half of them were hyperpolarized by mCPP or lorcaserin. These findings, along with the observation that adult inhibition of PVH Htr2c neurons increased food intake, corroborated the reactivation/deletion experiments and demonstrated that activation of PVH Htr2c promotes feeding. We further demonstrated that hyperpolarization of PVH neurons involved opening of postsynaptic K\textsubscript{ATP} conductance. Most surprisingly, however, neither pretreatment with anti-G\textsubscript{αq} antibodies nor a PLC inhibitor prevented Htr2c-mediated inhibition. Instead, it was blocked by the G\textsubscript{αi/o}-sensitive PTX. Although functional coupling between Htr2c and G\textsubscript{αi/o} has been described previously in Xenopus oocytes (Chen et al., 1994; Lucaites et al., 1996), we present evidence that this mechanism is important in the mammalian nervous system. These findings suggest that Htr2c can differentially regulate neuronal excitability and food intake in different neurons by coupling with distinct intracellular signaling events.

**Limitations of the study**

Our study utilizes sophisticated mouse genetic models in which Htr2c is selectively deleted or restored in Sim1::Cre neurons during early life. However, whether developmental manipulations of gene expression could have contributed to the observed phenotypes in adult mice remains unknown. We also cannot rule out the possibility that alterations in Htr2c expression in Sim1::Cre neurons may cause changes in gene transcription in these neurons or in other brain regions. Additionally, we use a Htr2c::Cre activated tdTomato reporter to mark Htr2c-expressing neurons in the PVH. Although we have validated this genetic tool in a previous study (Park et al., 2020), it may not capture the entire Htr2c neuron population. Finally, we use a chemogenetic approach to demonstrate that PVH Htr2c neurons are sufficient to drive feeding behaviors in vivo. Although this type of approach has been used in many studies, these manipulations may not recapitulate the normal physiological changes in the activity of PVH Htr2c neurons.

**STAR★METHODS**

**RESOURCE AVAILABILITY**

**Lead contact**—Further information and requests for resources and reagents should be directed to the lead contact, Chen Liu (chen.liu@utsouthwestern.edu).

**Materials availability**—No new unique reagents were generated in this study. Reagents will be made available upon completion of a Material Transfer Agreement.

**Data and code availability**

- All data reported in this paper will be shared by the lead contact upon request.
This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center and Korea Advanced Institute of Science and Technology.

Mice—All mice were housed in a temperature-controlled room with a twelve-hour-light/dark cycle (lights on at 06:00 a.m., lights off at 6:00 p.m.) in the animal facility of the University of Texas Southwestern Medical Center at Dallas. Food and water were supplied ad libitum. Mice were fed with either regular chow (#2916, Harlan-Teklad, Madison, WI; 4.25% kcal from fat) or a high-fat diet (HFD, #88137, Harlan Teklad, 42% kcal from fat). Htr2c^{RN/Y}; Sim1::Cre ±, Htr2c^{RN/Y}, and Htr2c^{+/Y}; Sim1::Cre ± mice were maintained on a C57BL/6 background, whereas Htr2c^{fl/Y}; Sim1::Cre and Htr2c^{fl/Y} mice were on a mixed C57BL/6 × 129 background. Htr2c::Cre mice were generated by conventional gene targeting in mouse embryonic stem cells.

METHOD DETAILS

Body weight and composition—Body weight was monitored weekly from weaning (four-week-old) to twenty-five weeks of age. In the HFD studies, mice were maintained on regular chow until six weeks old before being fed HFD. Body composition was assessed using the Bruker Minispec mq10 NMR analyzer at six and sixteen weeks of age.

Metabolic cage studies—Data for food intake, meal patterns, energy expenditure, and physical activities were collected using a combined indirect calorimetry system (TSE Systems GmbH, Bad Homburg, Germany). Experimental animals were individually acclimated in the metabolic chambers for five days before data collection. During data collection, mice were initially fed the chow diet in the first two days of data collection and switched to the HFD diet for the next three days in the metabolic cages.

Behavioral analyses—All tests were carried out in the UTSW Rodent Behavior Core. Elevated plus maze, dark light exploration, open field, and forced swim tests were conducted as previously described (Liu et al., 2010). At least two independent cohorts of mice were tested in all of the behavioral paradigms. All tests were conducted during the light cycle of the day between 10:30 am and 6:00 pm. All equipment was cleaned thoroughly with 70% ethanol between individual tests to remove odor cues. Individual behavioral experiments were performed at least forty-eight hours apart from one another. The tester/scorer was blind to genotype identification.

Response to d-Fen, mCPP, and lorcaserin—We measured food intake in overnight-fasted mice hourly (up to six hours) after an intraperitoneal dose of D-Fen (6 mg/kg; Sigma-Aldrich), mCPP (3 mg/kg; Sigma-Aldrich), lorcaserin (10 mg/kg, a gift from Dr. Kathryn A. Cunningham) or sterile saline. Drugs and saline were administered in a counterbalanced...
manner as previously described (Xu et al., 2008; Xu et al., 2010). Food was given to individually housed mice thirty minutes after treatment.

**Blood glucose, glucose, and insulin tolerance tests**—For GTTs, chow-fed weight-matched animals were fasted for 18 hours (starting at 3 p.m.) with water provided *ad libitum*. At 9:00 a.m. of the next day, blood glucose levels were monitored at 15, 30, 60, 90, and 120 minutes after an i.p. dose of glucose (1.5 g/kg body weight). For ITTs, chow-fed weight-matched mice were fasted for 2 hours with water *ad libitum* on the experiment day. Mice were given an i.p. dose of insulin (1 U/kg, Eli Lilly and Company, HI-210), and blood glucose levels were monitored at 15, 30, 60, 90, and 120 minutes after insulin injection. Blood glucose was analyzed using an AlphaTrak (Abbott Laboratories, North Chicago, IL) meter designed for use in rodents.

**Histology**—Mice were anesthetized with an overdose of chloral hydrate (500mg/kg, i.p.) followed by transcardial perfusion with 10% formalin (in DEPC-treated saline). Brains were collected and then subjected to a two-hour postfix with 10% formalin (in DEPC-treated saline) before being cryoprotected in 30% sucrose (in 1XPBS) overnight at 4°C. On the next day, twenty-five mm coronal brain sections were prepared with a freezing microtome. The free-floating sections were stored in cryoprotectant at −20°C until needed for immunohistochemistry or RNA *in situ* hybridization. For immunostaining, sections were stained with primary antibodies against oxytocin (Cat# ab2078, abcam), arginine vasopressin (Cat# PA5-19819, Thermo Scientific), tyrosine hydroxylase (Cat# ab112, abcam), c-FOS (Cat# sc-52-G Santa Cruz Biotechnology), and GFP (Cat# A6455, Life Technologies) followed by secondary antibodies with Alexa Fluor® 488 (Life Technologies) or DAB chromogen (DAKO) according to manufacturers’ protocols. *In situ* hybridization was performed as described previously (Vianna et al., 2012). Riboprobes were made from amplified PCR fragments with T7 and T3 promoters incorporated into the primer sequences so that the PCR product could be directly used as a template for the synthesis of the sense and antisense riboprobes.

**Electrophysiology**—Whole-cell patch-clamp recordings from Htr2c neurons were conducted as previously described (Sohn et al., 2011). Briefly, 3- to 5-week-old male or female mice were deeply anesthetized with i.p. injection of 7% chloral hydrate or isoflurane inhalations and trans-cardially perfused with a modified ice-cold artificial CSF (ACSF) (described below), in which an equiosmolar amount of sucrose was substituted for NaCl. The mice were then decapitated, and the entire brain was removed, and immediately submerged in ice-cold, carbogen-saturated (95% O₂ and 5% CO₂) ACSF (126 mM NaCl, 2.8 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 5 mM glucose). A brain block containing the hypothalamus was made. Coronal sections (250 μm) were cut with a Leica VT1000S or VT1200S Vibratome and then incubated in oxygenated ACSF at room temperature for at least 1 h before recording. Slices were transferred to the recording chamber and allowed to equilibrate for 10–20 min before recording. The slices were bathed in oxygenated ACSF (32°C–34°C) at a flow rate of ~4 ml/min. The pipette solution for whole-cell recording was modified to include an intracellular dye (Alexa Fluor 488) for whole-cell recording: 120 mM K-gluconate, 10 mM KCl, 10 mM...
HEPES, 5 mM EGTA, 1 mM CaCl₂, 1 mM MgCl₂, and 2 mM MgATP, 0.03 mM Alexa Fluor 488 hydrazide dye, pH 7.3. Epifluorescence was briefly used to target fluorescent cells, at which time the light source was switched to infrared differential interference contrast imaging to obtain the whole-cell recording (Zeiss Axioskop FS2 Plus equipped with a fixed stage and a QuantEM:512SC electron-multiplying charge-coupled device camera or Nikon Eclipse FN1 equipped with a fixed stage and an optiMOS scientific CMOS camera). Electrophysiological signals were recorded using an Axopatch 700B amplifier (Molecular Devices), low-pass filtered at 2–5 kHz, and analyzed offline on a PC with pCLAMP programs (Molecular Devices). Recording electrodes had resistances of 2.5–5 MΩ when filled with the K-gluconate internal solution. Input resistance was assessed by measuring voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse steps (500 ms of −10 to −50 pA or −5 to −25 pA). TTX, CNQX, AP5, picrotoxin, U73122, pertussis toxin, and 5-HT were obtained from Tocris. Anti-Gq antibody was obtained from Merck Millipore. Tolbutamide and glibenclamide were obtained from Sigma-Aldrich. All solutions were made according to the manufacturer’s specifications. Stock solutions of U73122 were made by dissolution in DMSO (Sigma-Aldrich). The concentration of DMSO in the external solution was maintained below 0.1%. Stock solutions of tolbutamide were made by dissolution in 100% ethanol, with the final ethanol concentration in ACSF less than 0.5%. Stock solutions of all other drugs were made by dissolution in distilled water. Membrane potential values were not compensated to account for junction potential (−8 mV).

**Stereotaxic injections**—Detailed experimental procedures were carried out according to a published protocol (Cetin et al., 2006). Briefly, six to eight-week-old Htr2c-Cre mice were deeply anesthetized by an i.p. dose of ketamine/xylazine. Mice were placed into a stereotaxic frame and a small opening was made in the skull directly over the injection site (bregma, AP, −0.7mm, DV, −4.5mm, ± 0.2mm from bregma). Coordinates for stereotaxic injections were obtained from the Paxinos mouse brain atlas. About 75-100 nL of AAV viruses that contain either stimulatory (hM3Dq) or inhibitory (hM4Di) DREADD constructs (AAV serotype 2 or 8, UNC viral core) were pressure injected into the PVH (unilateral or bilateral). After injection, all animals were allowed to recover for at least three weeks before being used for the feeding studies.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Replicate information is indicated in the figure legends. All results are given as mean ± SEM and analyzed by using statistical tools implemented in Prism (GraphPad version 9). Statistical analyses were performed using the Student’s t test and regular one-way or two-way analysis of variance (ANOVA). Differences with p < 0.05 were considered to be significant. p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***)

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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Highlights

• Selective restoration of Htr2c in Sim1 neurons promotes high-fat diet (HFD) feeding
• Selective deletion of Htr2c in Sim1 neurons reduces HFD feeding
• PVH Htr2c neurons bidirectionally regulate food intake
• Htr2c is coupled to the Gαi/o-K_{ATP} pathway in PVH neurons to inhibit their activity
Figure 1. Restoration of Htr2c only in Sim1 neurons increases food intake and body weight in male mice

(A) Body weight curves; F(28, 294) = 9.802, p < 0.001.

(B) Heat production during light (06:00–18:00) and dark (18:00–06:00) phases of a day; F(2, 24) = 1.961, p = 0.16.

(C and D) Food intake (C; F(2, 24) = 13, p < 0.001) and (D) respiratory exchange ratio (F(2, 24) = 1.712, p = 0.2) in HFD-fed male mice.

(E and F) Percentage of food intake (chow) after an i.p. dose of dFen (E, 6 mg/kg) or mCPP (F, 5 mg/kg), compared with vehicle-treated mice during the first 2 h of refeeding (F(2, 24) = 13.49 and F(2, 24) = 14.5 respectively; p < 0.001).

(G) Food intake (chow) after an i.p. dose of vehicle (saline) or lorcaserin during the first 2 h of refeeding; p = 0.05.

Values represent mean ± SEM; n = 8–11; two-way ANOVA with Tukey’s post hoc tests in (A)–(D); one-way ANOVA with Tukey’s post hoc tests in (E) and (F); paired t test in (G); *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 2. Selective deletion of Htr2cs in Sim1 neurons reduces food intake and body weight in male mice.

(A–D) In situ hybridization of Htr2c mRNA in the PVH (A and B) and amygdala (C and D) of Htr2c^fl/Y and Htr2c^Sim1KO mice.

(E) Body weight curves; F(15, 360) = 4.78, p < 0.001.

(F) Body composition; F(3, 96) = 3.91, p = 0.01.

(G) Food intake during light and dark phases of a day; F(1, 20) = 13.44, p = 0.002.

(H and I) Heat production (H; F(1, 20) = 0.33, p = 0.57) and (I) physical activity (F(1, 20) = 0.16, p = 0.7) in HFD-fed mice.

(J and K) Responses to dFen (J, 1 mg/kg) and mCPP (K, 5 mg/kg) in chow-fed Htr2c^fl/Y and Htr2c^Sim1KO mice.

Values represent mean ± SEM; n = 11–15, two-way ANOVA with Tukey’s post hoc tests in (E)–(I); unpaired t tests in (J) and (K); *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3. PVH Htr2c neurons directly regulate food intake in male mice
(A) Schematic for stereotaxic delivery of AAVs that contain stimulatory (hM3Dq) or inhibitory (hM4Di) DREADDs.
(B) Representative image showing unilateral targeting of GFP-labeled DREADD constructs in the PVH.
(C) Immunohistochemistry for Fos proteins.
(D) Double immunostaining for Fos and GFP. Arrowheads indicate double-labeled neurons.
(E) Bath application of CNO (5 μM) increased the firing rates of GFP-labeled PVH neurons.
(F) Change in membrane potentials.
(G) Stimulation of PVH Htr2c neurons suppressed fasting-induced refeeding; F(2, 36) = 3.511, p = 0.04.
(H) Food intake during the 1-h satiation period after fasting.
(I) Silencing of PVH Htr2c neurons promoted feeding; F(2, 28) = 4.832, p = 0.002.
Values represent mean ± SEM; *p < 0.05, **p < 0.01; two-way ANOVA with Sidak’s post hoc tests in (F), (G), and (I). Scale bars, 200 μm (B and C) and 20 μm (D).
Figure 4. Activation of Htr2c hyperpolarizes PVH Htr2c neurons

(A) Bath applications of mCPP caused prompt hyperpolarization of membrane potential, which was completely reversed by subsequent applications of tolbutamide.

(B) mCPP-induced hyperpolarization was not affected by addition of TTX and fast synaptic blockers in the bath solutions.

(C) Bath applications of lorcaserin, an Htr2c-specific agonist, caused prompt hyperpolarization of membrane potential. Downward deflections shown in the trace represent voltage responses to current steps, which are shown as insets below.

(D) Current-voltage (IV) relationship before (control) and after drug (lorcaserin) application. The E<sub>rev</sub> is close to the equilibrium potential for K<sup>+</sup> (E_K).

(E) Bar graphs summarizing the effects of tolbutamide on mCPP-induced hyperpolarization.

(F) Bar graphs summarizing the effects of glibenclamide.

(G) Bar graphs showing the average amplitude of mCPP-, lorcaserin-, or 5-HT-induced hyperpolarization.

(H) Table summarizing the numbers and percentages of the responses to mCPP treatment in recorded Htr2c neurons within the PVH.
Values represent mean ± SEM; *p < 0.05, ***p < 0.001; repeated-measures two-way ANOVA with Tukey’s or Bonferroni’s post hoc test in (E)–(G).
### KEY RESOURCES TABLE

| REAGENT                        | SOURCE                      | IDENTIFIER                   |
|--------------------------------|-----------------------------|------------------------------|
| **Antibodies**                 |                             |                              |
| Anti-oxytocin                   | Abcam                       | RRID: AB_302818, Cat# ab2078 |
| Anti-arginine vasopressin       | Thermo Scientific           | RRID: AB_11003086, Cat# PA5-19819 |
| Anti-tyrosine hydroxylase       | Abcam                       | RRID: AB_297840, Cat# ab112  |
| Anti-c-Fos                      | Santa Cruz                  | RRID: AB_2629503, Cat# sc-52-G |
| Anti-GFP                        | Life Technologies           | RRID: AB_221570, Cat# A6455  |
| Anti-Gq11α                      | Merck Millipore             | RRID: AB_310221, Cat# 06-709 |
| **Bacterial and virus strains**|                             |                              |
| hSyn-DIO-mCherry                | Addgene plasmid             | RRID:Addgene_50459, Cat# 50459 |
| hSyn-DIO-hM3D(Gq)-mCherry       | Addgene plasmid             | RRID:Addgene_44361, Cat# 44361 |
| hSyn-DIO-hM4D(Gi)-mCherry       | Addgene plasmid             | RRID:Addgene_44362, Cat# 44362 |
| **Chemicals, peptides, and recombinant proteins** |             |                              |
| Insulin (Humulin)               | Lilly                       | Cat# HI-210                  |
| Dextrose 50% Solution           | Hospira                     | Cat# 00409-6648-02           |
| D-Fen                           | Sigma                       | Cat #F112                    |
| mCPP                            | Sigma                       | Cat #C-089                   |
| Lorcanerin                     | A gift from Dr. Kathryn A. Cunningham | N/A                      |
| Chloral hydrate                 | Sigma                       | Cat #S0389                   |
| Sucrose                         | Sigma                       | Cat #SML2304                 |
| CNO                             | Sigma                       | Cat #I128                    |
| CNQX disodium salt              | Tocris                      | Cat #1045                    |
| DL-AP5                          | Tocris                      | Cat #0105                    |
| Picrotoxin                      | Tocris                      | Cat #1128                    |
| TTX citrate                     | Tocris                      | Cat #1069                    |
| Pertussis toxin                 | Tocris                      | Cat #1268                    |
| 5-HT                            | Tocris                      | Cat #3097                    |
| Tolbutamide                     | Sigma                       | Cat #T0891                   |
| Glibenclamide                   | Sigma                       | Cat #G0639                   |
| Rodent Diet With 42 kcal% Fat   | Harlan Teklad               | Cat# 88137                   |
| Standard chow                   | Harlan Teklad               | Cat# 2916                    |
| **Experimental models: Organisms/strains** |             |                              |
| Mouse: C57BL/6J                 | The Jackson Laboratory      | RRID: JAX:000664             |
| Mouse: Sim1::Cre                | Bradford B Lowell           | RRID: MGI:3692526            |
| Mouse: Htr2c::Cre               | Chen Liu                    | Park et al., 2020            |
| Mouse: Htr2e<null>              | Joel K Elmquist             | RRID: MGI:3841486            |
| Mouse: Htr2e<lox>               | Joel K Elmquist             | RRID: MGI:5569357            |
| Mouse: Gt(Rosa)2f6Sert<BRCl<dtL>D(Ai14) | The Jackson Laboratory     | RRID: JAX: 007914             |
| **Oligonucleotides**            |                             |                              |
| REAGENT                                      | SOURCE              | IDENTIFIER         |
|----------------------------------------------|---------------------|--------------------|
| See Table S1 for oligo nucleotide information | N/A                 |                    |
| Other                                        |                     |                    |
| Indirect calorimetry system – TSE PhenoMaster | TSE Systems Germany | N/A                |
| EchoMRI                                      | EchoMRI Corporation | N/A                |
| Vibratome                                    | Leica               | N/A                |
| Glucometer                                   | AlphaTrak           | N/A                |
| GraphPad version 9                           | GraphPad Software Inc | https://www.graphpad.com |